#14
RW

PATENT APPLICATION

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January 13, 2003
Date

Lara Russell
Lara Russell

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Sprecher, Cindy A., Novak, Julia E., West, James W., Presnell, Scott R., Holly, Richard D., and Nelson, Andrew J.
Application No.	:	09/825,561
Filed	:	April 3, 2001
For	:	SOLUBLE ZALPHA11 CYTOKINE RECEPTORS
	Examiner :	Ruixiang Li
	Art Unit :	1646
	Docket No. :	00-22

Declaration Under 37 CFR § 1.131

Sir:

We, Julia E. Novak, Cindy A. Sprecher, James W. West, Scott R. Presnell, Richard D. Holly, Andrew J. and Nelson, and Julia E. Novak, hereby declare as follows:

1. We are the named inventors on the above-identified application and have reviewed and understand the specification and claims of the above-identified application.

2. All of the work described herein and illustrated by the attached Exhibits was performed in the United States under our direction.

3. I have read and understood the reference cited by the Office, Presnell et al., WO 00/17235, published March 30, 2000.

4. Exhibit 1 comprises copies of pages and figures describing data from a draft manuscript prepared by one of us (Julia E. Novak) submitted in-house for review prior to March 30, 2000. Figure 3 comprised a summary of the data prepared by one or more of us (James E. West and Andrew J. Nelson) whose work is described in detail below and provided in Exhibits 2 and 3. This draft manuscript establishes that the invention of subject matter of the above-identified application, i.e., heterodimeric and multimeric zalpha11-containing receptors, occurred prior to March 30, 2000, the publication date of the cited reference.

5. Exhibit 1 (including figure legend "Figure 3." and accompanying Figures 3A and 3B) includes experimental data that establishes the invention of subject matter of the above-identified application, i.e., heterodimeric zalpha11-containing receptors, prior to March 30, 2000. Please note that at the time this manuscript was written we initially used "IL-19R," as opposed to the published "IL-21R" as nomenclature for the zalpha11 receptor polypeptide. Similarly, the nomenclature "IL-19" was used for the ligand (zalpha11Ligand), as opposed to the published "IL-21." Assays were designed and carried out to test whether zalpha11Ligand (IL-21) might in addition to the IL-21R (zalpha11) use the IL2 receptor common gamma chain (γc), a.k.a. IL-2Rγ, as part of its receptor complex, since the cytokines most closely related to zalpha11Ligand (IL-21) utilize this subunit.

(a) ORIGEN Assay Data (see enclosed Figure 3A, and legend). The ORIGEN electrochemiluminescence (ECL) technology (Igen, Inc.) provided a method for measuring dimerization of two differentially labeled proteins in the presence of an unlabeled third protein. A ruthenium metal chelate (Ru) label on one protein will emit luminescence when brought near an electric field through dimerization with a biotinylated protein which is held in place via streptavidin-coated magnetic beads. Using this assay, we assessed the dimerization of the soluble zalpha11 receptor (IL-21R; denoted as IL-19R in Exhibit 1) with various soluble class I cytokine receptor subunits in the presence of the zalpha11Ligand, IL-21 (denoted as IL-19 in Exhibit 1). Homodimerization of the zalpha11 receptor (denoted as IL-19R in Exhibit 1) did not occur in the presence of the zalpha11Ligand (IL-21; denoted as IL-19 in Exhibit 1) using this assay. However,

in this assay, ligand-mediated dimerization of Ru-IL21R (denoted as Ru-sIL19R in Exhibit 1) with bio-IL21R (denoted as bio-sIL19R in Exhibit) or of Ru-IL2Rγ (Ru-sγc) with bio-IL21R (denoted as bio-sIL19R in Exhibit) was measured. The results of this assay showed that zalpha11 receptor (IL-21R; denoted as IL-19R in Exhibit 1) and IL-2Rγ dimerized in the presence of IL21, but not IL2, IL4, or IL15 (Figure 3A); hence the dimerization of zalpha11 receptor (IL-21R; denoted as IL-19R in Exhibit 1) and IL-2Rγ was specific to IL21.

(b) Costimulation Data (see enclosed Figure 3B, and legend). In addition to the ORIGEN assay described above, experiments were conducted in order to determine whether the dimerization of zalpha11 receptor and IL-2Rγ was necessary for signal transduction, neutralizing monoclonal antibodies to IL-2Rγ (anti-γc antibodies) were used in proliferation assays with normal murine splenic B cells. The addition of the anti-γc antibodies TUG/m2 and 3E12 partially blocked proliferation induced by IL21 (denoted as IL-19 in Exhibit 1) and anti-CD40 (Figure 3B), suggesting that IL-2Rγ plays a role in IL21 signal transduction in B cells. This data further supported that the zalpha11 receptor forms a functional complex with the IL2 receptor common chain (γc), a.k.a. IL-2Rγ.

(c) Multiple subunits. At least one of us (Julia E. Novak) recognized prior to March 30, 2000 that the IL2 receptor had been studied in detail and is composed of an α-β-γc heterotrimer. The β and γc subunits are both essential for signal transduction and are members of the hematopoietin receptor superfamily, whereas the α subunit appears to primarily be involved in high-affinity binding conversion and is structurally distinct from the hematopoietin receptor family. The γc subunit has been shown to participate in forming the receptors for IL4, IL7, IL9, and IL15, in addition to IL2 (for review, see Sagamura, K. et al., Ann. Rev. Immunol. 14: 179-205 (1996); copy enclosed)). Based on what was known about other Class I cytokine receptors, we recognized prior to March 30, 2000 that not only could zalpha11 receptor form a heterodimeric complex with γc as we had demonstrated, but that it would not be unreasonable to form a trimeric or

multimeric complex, for example, comprising other Class I cytokine receptor subunits, for example, in addition to the IL-2Rγ receptor.

6. The experiments summarized in Exhibit 1, performed prior to March 30, 2000, describe and provided experimental evidence for a functional zalpha11 receptor complex that contains the IL2 receptor common chain (γc), a.k.a. IL-2Rγ. Our data suggested that IL21 acts through a receptor complex that includes zalpha11 receptor and the γc subunit of IL2R, even though the cytoplasmic domain of zalpha11 receptor was capable of transducing signal in a homodimeric configuration (e.g., see Novak et al., US Patent No. 6, 307, 204; cited by Office). This finding was similar to the known receptor, IL4Rα, which is also capable of signaling as a homodimer (Kammer, W. et al.., J. Biol. Chem. 271: 23634-23637 (1996); copy enclosed), although the natural functional IL4 receptor complex is a IL4Rα/γc heterodimer.

7. Exhibit 2 comprises copies of notebook pages 42 and 103 from ZymoGenetics Notebook #6917, and pages 130, 138-142 from ZymoGenetics Notebook #6637, describing data prepared by one of us (James W. West) prior to March 30, 2000. The following experiments were designed to ask whether IL2Rγ was a component of the zalpha11 receptor complex, which binds zalpha11Ligand (IL21). We used an ORIGEN dimerization assay as described in paragraph 5(a) above.

(a) Pages 42 and 103 from ZymoGenetics Notebook #6917. These pages show an ORIGEN assay where IL21 (denoted as "IL-19", "zalpha11Lig" or "α11lig" in the notebook) promoted the dimerization of the zalpha11 receptor (denoted in shorthand as "zα11" in the notebook) and IL2Rγ. In contrast IL2, IL4 and IL15 did not demonstrating specificity. These experiments were summarized in the data graph as shown at the bottom of page 103 (this was the graph used for figure 3A in Exhibit 1). The results of this assay showed that zalpha11 receptor and IL2Rγ dimerized in the presence of IL21, but not IL2, IL4, or IL15 (Figure 3A); hence the dimerization of zalpha11 and IL2Rγ was specific to IL21.

(b) Pages 130, 138-142 from ZymoGenetics Notebook #6637. Previous experiments to those described above (Paragraph 7(a)) using the same ORIGEN dimerization assay described herein showed that IL-21 (denoted as "zalpha11Lig"

or "α11lig" or "zalpha11 ligand" in the notebook) in conditioned media from clones expressing IL-21 promoted dimerization of zalpha11 and IL-2Rγ. Page 130, and 138-142 shows that 40X concentrated IL-21 containing media prepared by one of us (Cindy A. Sprecher) from clones expressing "zalpha11 ligand" (Page 130) promoted the dimerization of zalpha11 receptor and IL-2Rγ in the ORIGEN dimerization assay. Pages 138-139 show that the receptor dimerization assay results showed heterodimerization of zalpha11 receptor and IL-2Rγ in the presence of zalhpa11Ligand conditioned media. Pages 140-141 showed a repeat of these results showing heterodimerization of zalpha11 and IL-2Rγ in the presence of IL-21 ("zalpha11 lig"). The ORIGEN electrochemiluminescence (ECL) technology (Igen, Inc.) provided a method for measuring dimerization of two differentially labeled proteins in the presence of an unlabeled third protein. A ruthenium metal chelate (Ru) label on one protein will emit luminescence when brought near an electric field through dimerization with a biotinylated protein which is held in place via streptavidin-coated magnetic beads. Using this assay, we assessed the dimerization of zalpha11 receptor with various class I cytokine receptor subunits in the presence of the IL-21 (zalpha11Ligand). Homodimerization of zalpha11 receptor did not occur in the presence of IL-21 (zalpha11Ligand) using this assay, nor did heterodimerization with IL-4R or with IL-4R and IL-2Rγ. However, in this assay, ligand-mediated dimerization of zalpha11 with bio-IL-2Rγ was shown.

8. Exhibit 3 comprises copies of notebook pages 36-41 from ZymoGenetics Notebook #7072 describing data prepared by one of us (Andrew J. Nelson) prior to March 30, 2000. These notebook pages have the raw data and results of experiments used to support the "Costimulation Data" described in Figure 3B, and legend in Exhibit 1. Pages 36 and 37 described the isolation of splenic B-cells, pre-incubation with the anti-γc antibodies (TUG/m2 and 3E12) and co-stimulation of the B-cells with IgM or andt-CD40 antibodies in the presence of either murine zalpha11-Ligand (a.k.a., IL-21 or "IL-19" as described above), mIL2, mIL4 or

mIL15 at concentrations listed. Sixteen hours prior to harvesting, 1 μCi ^{3}H-thymidine (Amersham, Piscataway, NJ) was added to all wells to assess whether the B-cells had proliferated. The raw data from the TopCount Microplate Scintillation Counter (Packard) showing ^{3}H-thymidine-incorporation and hence proliferation of the B-cells in the presence of the various cytokines is shown on page 37-38; and the corresponding summary via graphic representations of each experiment are presented on pages 38-41. Specifically, the results shown on page 38 ("7072.36 anti CD40 w/ titrating amounts of zalpha11Lig w/wo Rx of TUG & 3E12 cells CD19 pos select from frozen PBMC") showed that the addition of the anti-γc antibodies partially blocked proliferation induced by the zalpha11-Ligand (IL-21) and anti-CD40 (also shown in Figure 3B in Exhibit 1), which suggested that the IL2 receptor common chain (γc), a.k.a. IL-2Rγ.played a role in zalpha11-Ligand (IL-21) signal transduction in B cells. This data further supported that the zalpha11 receptor formed a functional complex with the IL2 receptor common chain (γc), a.k.a. IL-2Rγ.

9. The data summarized in Exhibits 1-3 showed the conception of heterodimeric and multimeric zcytor11-comprising receptors, as well as the actual reduction to practice of at least one heterodimeric receptor complex containing an isolated soluble receptor polypeptide comprising a sequence of amino acid residues as shown in SEQ ID NO:6 (zalpha11 soluble receptor), wherein the soluble receptor polypeptide forms a heterodimeric receptor complex; as well as the receptor complex further comprising other Class I cytokine receptor subunits, for example, the IL-2Rγ receptor.

10. On the basis of these Exhibits, which document activities within the United States of America, we conclude that the invention described in claims 31-33, 35, 37, and 48-52, of the above-captioned application was conceived and reduced to practice prior to March 30, 2000.

11. We further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

By _____ Date 1/13/2003
Julia E. Novak

By _____ Date _____
Cyndi A Sprecher

By _____ Date _____
James W. West

By _____ Date JAN 13, 2003
Scott R. Presnell

By _____ Date 1-13-03
Richard D. Holly

By _____ Date 1/13/03
Andrew J. Nelson

11. We further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willfully false statements and the like is punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and may jeopardize the validity of any patent issuing from this patent application.

By _____ Date_____

Julia E. Novak

By _____ Date_____

Cyndi A Sprecher

By _____ Date 1/12/03

James W. West

By _____ Date_____

Scott R. Presnell

By _____ Date_____

Richard D. Holly

By _____ Date_____

Andrew J. Nelson

FIGURE LEGENDS

Figure 1. Conditioned media from cDNA pools stimulates proliferation of BaF3/IL19R cells. The four pools selected for breakdown are shown; other positive pools gave similar results. CM were used at 25% of total assay volume. Strong proliferation was seen when samples were assayed without soluble receptor (black bars); the addition of IL19R soluble receptor (0.5 μg/ml) completely neutralized the activity (striped bars). Unstimulated BaF3/IL19R cells alone are shown as background (open bars).

Figure 2. Alignment of human and murine IL19 with related cytokines. Identities (:) or similarities (*) between either human or murine IL19 and human IL15 are shown. Mature amino termini are indicated by open boxes. Potential N-linked glycosylation sites are underlined.

Figure 3. IL19 receptor complex. (A) Association of sIL19R and sγc is mediated by IL19. In the presence of IL19, Ru-sγc and Bio-sIL19R associate, giving rise to a strong luminescent signal (open bars). The specificity of this association is demonstrated by the lack of luminescence in the presence of IL2, IL4 or IL15 (open bars). The filled bars illustrate that none of the cytokines tested mediate homodimerization of IL19R. (B) Requirement for γc during IL19 costimulation of murine B cells. B cells were purified from the spleens of C57Bl/6 mice and cultured with 0-30 ng/ml IL19 and 1 μg/ml anti-CD40 mAb either without (filled bars) or with (open bars) 50 μg/ml each of the anti-γc mAbs TUG/m2 and 3E12 (PharMingen International). Values plotted represent the mean value (±SD) obtained from triplicate culture. These results represent those obtained in three independent experiments.

Figure 3A

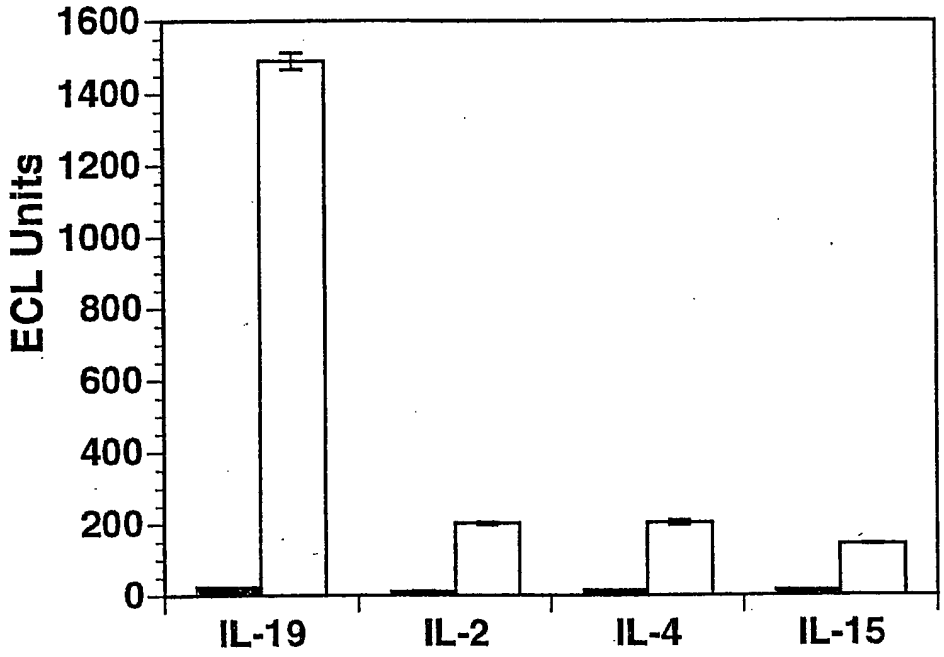
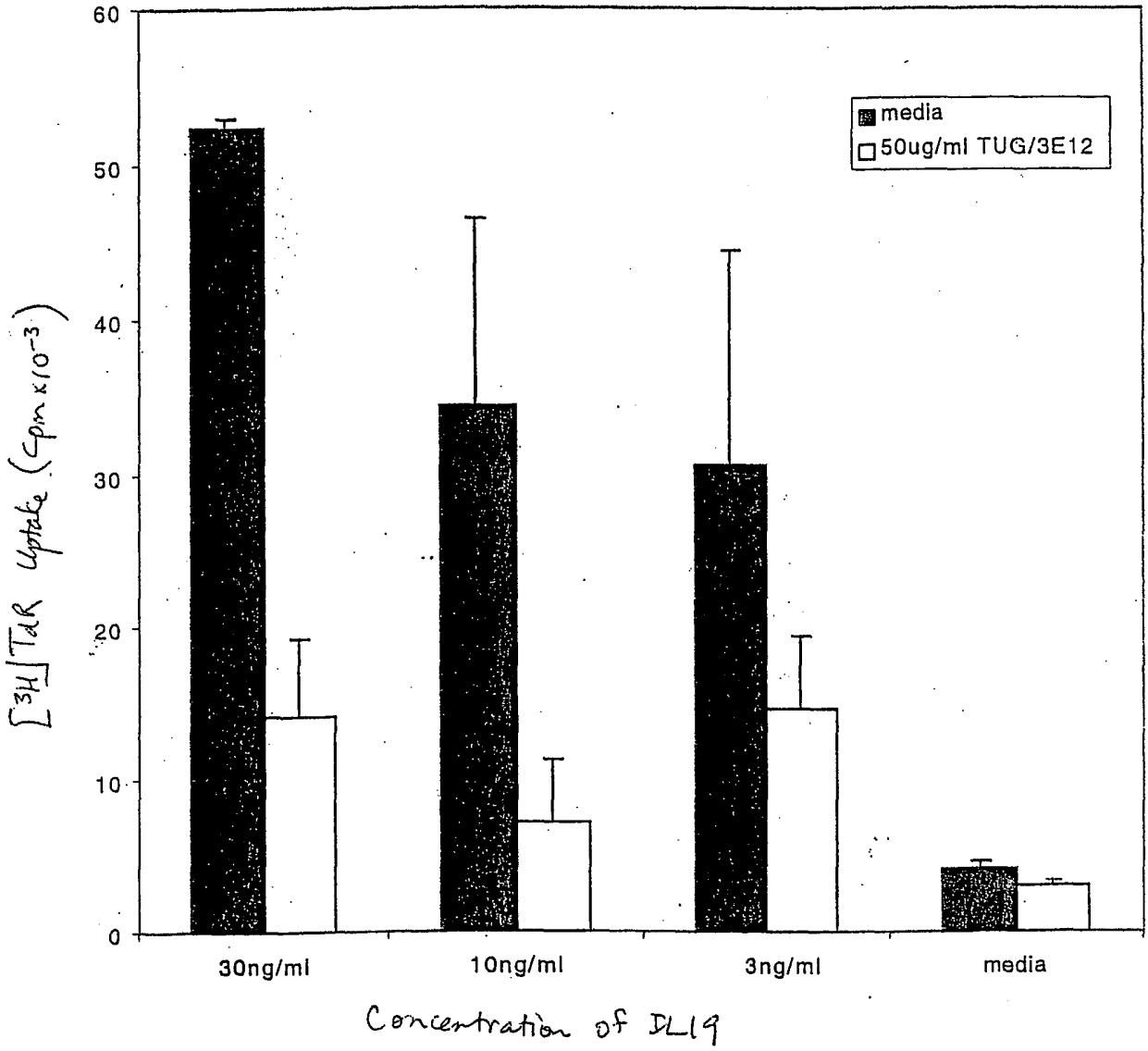


Fig. 3(B)

B cells - Array

Abs from PharMingen International



Mouse B cells

with α-CD40

6917.42 Book # 6917

Project No.

42 Book No. TITLE Ru Labeling

From Page No. [redacted]

75 µg NHS-TAG – 71 nmols 75 µl ≡ 0.95 nmols/µl
DMSO

					TAG/protein
100 µg	IL2RG 40kDa	2.5 nmols	+ 20 µl NHS-TAG	19 nmols	7.6
	450 µl H2O + 50 µl 1M NaCarb 8.5				
50 µg	IL6sR 38kDa	1.3 nmols	+ 10 µl NHS-TAG	9.5 nmols	7.3
	435 µl H2O + 50 µl 1M NaCarb 8.5				
351 µg	zCytoR7CF 50kDa	7 nmols	+ 45 µl NHS-TAG	42.7 nmols	6.1
	1 mL PBS + 100 µl NaCarb pH 8.5				

2x rhIL-2 sRγ
Interleukin 2 soluble Receptor Gamma recombinant human (Sf 21-derived)
Catalog Number 384-RG/CF
50.0 µg Lot KT017031
>97% Purity Store at -20°C
FOR RESEARCH USE ONLY
R&D SYSTEMS Minneapolis, MN 55413 1-(800)-343-7475
CARRIER FREE

2x rhIL-6 sR
Interleukin 6 soluble Receptor recombinant human (Sf 21-derived)
Catalog Number 227-SR/CF
25.0 µg Lot DG088041
>97% Purity Store at -20°C
FOR RESEARCH USE ONLY
R&D SYSTEMS Minneapolis, MN 55413 1-(800)-343-7475
CARRIER FREE

COPY

ORIGENS
TAG NHS-Ester™
Amount: 75 µg
Storage: -20°C Desiccated
IGEN International, Inc. 16020 Industrial Drive Gaithersburg, MD 20877

Incubate 2hrs RT in DARK & Rocking

Purify by PD10 chromatography

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by	[redacted]

Exhibit 2 2 of 8

Book #6917

TITLE ______ Project No. ______ Book No. ______

From Page No. 6637.132-142, 6917.42

The purpose of this exp is to determine specificity of zA11/IL2Rγ for binding ligands in the Alpha format. Origen assay (6637.142) was used.

Assays were done as described (6637.142)

Receptor – Bio zA11CEE, Ru-IL2Rγ.

Final Conc of receptor components 400ng/ml each (100μl volume) and 100μl of cytokine solution from 300 ng/ml → 133pg/ml 3 fold dilution

300
100
33.3 3 fold dilution
11.1
3.7
1.23
0.42
0.133

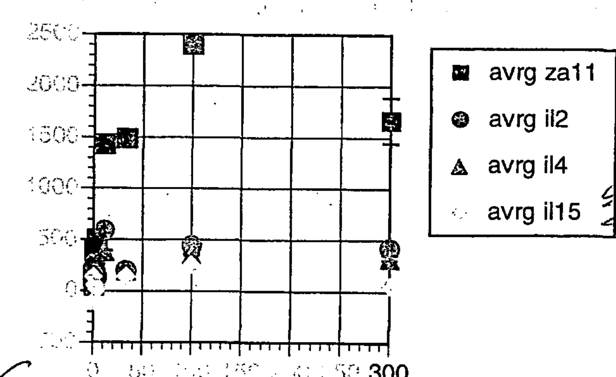
Incubate 1 hour
Add 3 μg M280 Beads (strept) in 50μl TBS.B
Incubate 1hr
Add 200μl Assay buffer & Read assay!

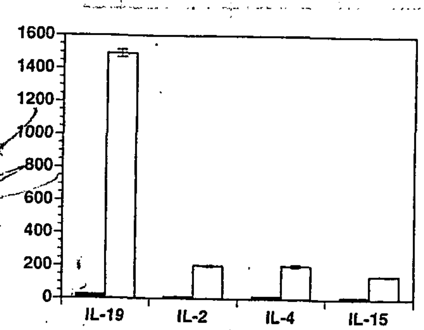
Label	a11/il2				avrg za11	std
1		300	4672.855	3867.554	4270.2045	569.4337979
2		100	5058.111	4957.878	5007.9945	70.875433998
3		33.3	4135.836	4045.337	4090.5865	63.99245659
4		11.1	3980.127	4083.524	4031.8255	73.11271985
5		3.7	3098.506	3103.617	3101.0615	3.614022758
6		1.23	3014.25	2966.127	2990.1885	34.02809963
7		0.42	2563.141	2745.109	2654.125	128.67080675
8		0.133	2774.374	2712.423	2743.3985	43.80597220

			avrg il2	std
3017.469	3033.324	3032.073	3027.622	8.814976290
3007.279	2993.934	3137.837	3046.35	79.51053787
2734.943	2844.285	2825.74	2801.656	58.51450703
3095.909	3191.006	3289.839	3192.251333	96.97099755
2733.048	2925.212	2551.224	2736.494666	187.0178217
2838.586	2832.151	2931.505	2867.414	55.59761251
2654.571	2700.159	2780.672	2711.800666	63.85148277
2769.608	2860.276	2824.689	2818.191	45.88193908

			avrg il4	std		
2780.25	2828.536	3183.673	2930.819666	220.3043135	2936.292	2699.088
3051.874	2866.943	2954.053	2957.623333	92.51718300	2862.978	2800.071
2694.708	2883.729	2831.59	2803.342333	97.62621505	2693.679	2757.384
3008.919	2986.001	2979.651	2991.523666	15.39574101	4284.784	4275.962
2927.355	2811.015	2679.572	2805.980666	123.9681900	2674.534	2660.46
2684.028	2647.047	2672.022	2667.699	18.88570531	2601.933	2761.453
2612.599	2762.233	2579.234	2651.355333	97.46126682	2456.573	2454.697
2710.03	2813.246	2851.711	2791.662333	73.26504924	2721.651	2767.603

	avrg il15	std
2345.135	2660.171666	297.4937149
2908.675	2857.241333	54.52879250
2779.43	2743.497666	44.53011554
4284.401	4281.715666	4.986500008
2517.221	2617.405	87.04679638
2615.954	2659.78	88.33004192
2592.656	2501.308666	79.11467199
2736.576	2741.943333	23.44147555





Concl:
Only α11 lig is an agonist of zA11/IL2Rγ -

To Page No. ______

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by [signature]	[redacted]

COPY

Notebook 6637

Project No.______

 Book No.______ TITLE Zα11 Ligand

From Page No. [redacted]

Got 3 cell lines from Cindy Sprecher. These cells are expressing Zα11 Ligand by BAF3 Assay & By Dimerization with hIL20 receptor in Origen Assay

Split cells into 20 T162 flasks EACH.

Refeed [redacted]
CHange medium to SF; SLV4 [redacted]
Harvest medium [redacted] & concentrate 40X on 5K cutoff filter and filter sterilize on 0.22 μm filter

Test for activity 6637.138, 140, 142

To Page No.

Witnessed & Understood by me,	Date	Invented by / Recorded by	Date
		(signature)	[redacted]

Exhibit 2 4 of 8

COPY 6637

138 Project No.______ Book No.______ TITLE ZαII Dim

From Page No. [redacted]

① 20x Receptor Bio-ZαII CF₅ 400 μg/ml 1ml TBS-B + 10 μl 400 μg/ml BioZαII

② 20x Bio IL-4α CF 4 μg/ml 1ml TBS-B + 4 μl 1mg/ml Bio IL4α

③ 20x Ru IL28 4 μg/ml 920 μl TBS-B + 80 μl 50 μg/ml Ru IL28

④ 20x Ru ZαII CEE 4 μg/ml 968 μl TBS-B + 32 μl 125 μg/ml Ru ZαII

Ⓐ 50 μl 1 — 20 μl/Assay pt
50 μl 3
100 μl TBS-B
Ⓑ 50 μl 1
50 μl 4
100 μl TBS-B
Ⓒ 50 μl 2
50 μl 3
100 μl TBS-B
Ⓓ 50 μl 2
50 μl 4
100 μl TBS-B
Ⓔ 50 μl 1
50 μl 2
50 μl 3
50 μl TBS-B
Ⓕ 50 μl 1
50 μl 2
50 μl 4
50 μl TBS-B
Ⓖ 50 μl 1
50 μl 2
50 μl 3
50 μl 4

— media PLA1011 PLA3H9 — cultured cells to confluence add SCV4 SF media & culture 3 days. Concentrate media on 5k Filter 40X.

80 μl media + 20 μl Receptor mix.
Incubate 1hr RT shaking
Add 6 μg Strept M280 Beads in 50 μl TBS
~~Incubate~~ 1hr RT shaking
Add 200 μl Assay buffer & Read

80mls → 2mls for PLA1011 & "—"

160mls → 4mls for PLA3H3

Conclusion:

① Everywhere that IL-2R~~o~~ & ZαII co-exist in the presence of PLA1011 or PLA3H9 but not Neg control there is "+" signal.

② Unlabeled IL2R8 Does not stimulate RuZαII/ZαIIBio Homodimerization in the presence of "+" media. Suggesting it's a simple dimerization of IL2R8, ZαII.

③ IL4R8 seems to reduce Ru IL2R8/ZαII.Bio dimerization.

6637.140

6637.139
To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
		Recorded by [signature]	[redacted]

COPY 6637

Project No.

TITLE Book No. 139

From Page No. 6637.132

Experiment 1, Data File: 6637138

Assay buffer reading = 6332

Tb	Ix	ECL	Dark/ABCur	LED/
1	1	2825	26	
			6369	
2	2	2730	28	
			6369	
3	3	2825	30	
			6369	
4	4	2824	30	
			6369	
5	5	3289	27	
			6281	
6	6	2488	32	
			6423	
7	7	4148	32	
			6369	
8	8	4046	26	
			6544	
9	9	2334	28	
			6369	
10	10	3046	31	
			6369	
11	11	3859	32	
			6369	
12	12	3596	30	
			6369	
13	13	5486	28	
			6369	
14	14	6175	35	
			6281	
15	15	10132	27	
			6369	
16	16	9674	31	
			6456	
17	17	3233	29	
			6281	
18	18	2809	27	
			6281	
19	19	2344	28	
			6425	
20	20	2240	28	
			6369	
21	21	4190	28	
			6369	
22	22	3595	30	
			6369	
23	23	6035	32	
			6369	
24	24	5840	28	

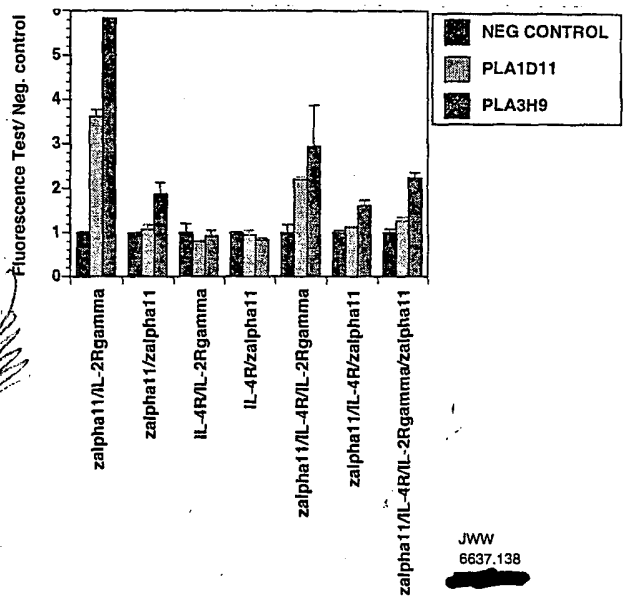
Experiment 1, Data File: 6637138

			6369
25	25	4271	34
			6456
26	26	4112	29
			6369
27	27	7734	34
			6369
28	28	7062	33
			6369
29	29	8002	31
			6281
30	30	16171	33
			6456
31	31	5801	31
			6281
32	32	4776	30
			6369
33	33	2926	32
			6374
34	34	2400	33
			6369
35	35	3308	31
			6369
36	36	3557	26
			6369
37	37	6162	30
			6456
38	38	9673	38
			6281
39	39	6331	33
			6456
40	40	5689	29
			6369
41	41	13513	34
			6369
42	42	12533	34
			6369

Conclusions:

(4) Minimal Homodimerization of zalpha11/zalpha11Fc in the presence of PLA1D11 or PLA3H9 both Ref. Neg control medium.

(5) PLA1D11 & PLA3H9 cell Cross express zalpha11 Ligand.



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140 Project No.______ Book No.______ TITLE ZK11/IL2R8 ?

From Page No. [redacted] 6637.138

	[stock]	Tube #	Total Volum	Diluent	V. Stock		[final]
20x Ru IL28	50 μg/mL	1	1 mL	TBS-B	+ 80 μl	Ru IL28	4 μg/mL
20x Bio ZK11CF	400 μg/mL	2	1 mL	TBS-B	+ 10 μl	Bio ZK11	4 μg/mL
20x Ru ZK11CF	125 μg/ml	3	1 mL	TBS-B	+ 32 μl	Ru ZK11 CF	4 μg/ml
20 IL-2sR8	500 μg/ml	4	1 mL	TBS-B	+ 8 μl	IL2R8 R&D Lot# KT016071 Cat# 324-RG/CF	4 μg/ml

Soln

(A) 40 μl 1 Ru-IL2R8
40 μl 2 Bio-ZK11
80 μl TBSB

(B) 40 μl 2 Bio-ZK11
40 μl 3 Ru-ZK11
80 μl TBS-B → 20 μl to Each tube

(C) 40 μl 2 Bio ZK11
40 μl 3 Ru ZK11
40 μl 4 IL2R8
40 μl TBS-B

(D) 40 μl 1 Ru-IL2R8
40 μl 2 Bio-ZK11
80 μl 4 IL2-R8 - 400 ng/ml
0.5 μl 500 μg/ml IL2R8 1.56 μg/ml (10x)

Run assay as described 6637.138

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Exhibit 2 7 of 8

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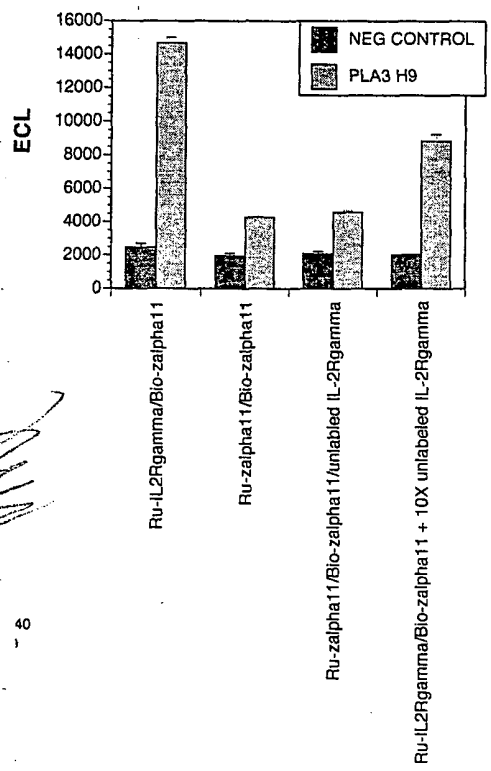
TITLE Book No. 141

From Page No. 6637.140

Experiment 1, Data File: 6637140

Assay buffer reading = 6808

Tb	Ix	ECL	Dark/ABCur	LED/C
1	1	2737	31 6369	
2	2	2345	41 6456	
3	3	2281	31 6369	
4	4	1993	28 6369	
5	5	2083	34 6475	
6	6	1771	34 6281	
7	7	2172	33 6281	
8	8	1883	29 6281	
9	9	2120	33 6369	
10	10	2031	34 6369	
11	11	14995	31 6369	
12	12	14315	35 6369	
13	13	14708	32 6369	
14	14	4291	33 6369	
15	15	4202	32 6369	
16	16	4324	31 6369	
17	17	4693	32 6456	
18	18	4459	31 6281	
19	19	4577	34 6456	
20	20	9194	35 6369	
21	21	8885	34 6281	
22	22	8405	36 6369	



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(cont'd)

zalpha receptor is zalpha IL2Rγ

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Solution A 6637.140

Combine 20µl Solution A with either 80µl neg control media or PLA3 H9 media for one hour, 45 min, 30 min & 15 min add 3µg M280 strep beads in 25µl TBS-B & incubate 30mm.

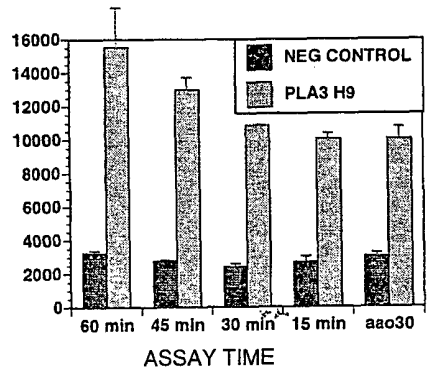
When beads were added additional tubes were set up by combining 80µl either control media or PLA3 H9 media with Solution A and beads. Tubs were incubated 30mm.

200µl Assy buffer was added and tube read.

Experiment 1, Data File: 6637142

Assay buffer reading = 6564

Tb	Ix	ECL	Dark/ABCur	LED/C
1	1	C 3344	35	
2	2	C 3124	6369	
3	3	H9 17203	36	
4	4	H9 13861	6369	
5	5	C 2717	38	
6	6	C 2792	6456	
7	7	H9 13503	40	
8	8	H9 12442	6286	
9	9	C 2533	37	
10	10	C 2276	6369	
11	11	H9 10871	42	
12	12	H9 10799	6281	
13	13	C 2438	41	
14	14	C 2931	6369	
15	15	H9 9796	40	
16	16	H9 10274	6281	
17	17	C 2844	40	
18	18	C 3175	6456	
19	19	H9 10537	39	
20	20	H9 9519	6369	
			36	
			6456	
			36	
			6287	
			33	
			6369	
			36	
			6293	
			38	
			6369	
			38	
			6281	
			35	
			6456	
			37	
			6281	
			36	
			6369	
			35	
			6456	



Concl: This assay can be done in 30 minutes by combining all Mgents & incubating for 30mm.

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Project No. [redacted]
Book No. 1072 TITLE Effect of TVb+3E12 w/2alpha11

From Page No.___

[redacted]

7-57 B/6 Spleens mesh tease, water lyse (pH) count
$175 \times 10^6 \rightarrow 1.6$ml in MAC Buffer

CD43 Miltenyi microbead RX. Add 400ul washed CD43 microbeads to cells, incubate 15 minutes iced
Wash MAC Buffer 1x. Run over washed LS/VS (2) columns in MAC Magnetic Holder. Wash through with 10ml MAC Buffer. Fall through Cells CD43 depleted. Cells Retained Column $CD43^+$

CD43 depleted $75 \times 10^6 \rightarrow 7.5$ml ($10 \times 10^6$/ml)

RX Cells - Preincubation cells

	Final	Working	10×10^6
TVb 188400 Pharm 1mg/ml	50ug/ml	200ug/ml	1ml + 1ml cells > 5ml
3E12 183700 Pharm 1mg/ml	50ug/ml	200ug/ml	1ml + 1ml > 5ml
TVb + 3E12	50 + 50	200 + 200	1ml + 1ml cells > 5ml

Mix together cells and antibodies. Incubate iced 1 hr.

Preincubation:	Final	Stock
M-2alpha11 Lg .176 mg/ml @ 1:100 = 1.76ng/ul	3 ng/ml	12
	10	40
	30	120
	media	

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TITLE

Book No.

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	Final	Working
M/L2 #41 10ug/ml	3 ng/ml	12
	10	40
	30	120
M/L4 #45 10ug/ml	3ng/ml	12
	10ng/ml	40ng
	30	120
M/L15 #62 10ug/ml	3ng/ml	12
	10ng/ml	40ng
	30ng/ml	120
anti-mCD40 second 1ng/ml	1ug/ml	4ug/ml
anti IgM Biosource 600ng/ml	10ug/ml	40ug/ml

PID: 767 ASCII File: C:\Data\Janet\DATA.101 Acquired: 1/14/00 12:30:15 PM Plate Temperature: 19.0C (66.2F)

Repeat 1: Sample CPMA 3 ng/ml 10 ng/ml 30 ng/ml

media (1)

	1	2	3	4	5	6	7	8	9	10	11	12	
A	15307	42159	34413	45488	21292	36212	51879	53083	52267	4021	3859	4678	2a/pbs
B	24670	26112	30330	22222	12029	22632	41498	40447	37361	4569	2891	4127	
C	40100	36253	32764	31664	17657	35340	41963	44443	52087	5472	6911	6167	
D	11373	20134	12347	10657	2534	8233	13531	19562	9420	3287	2636	3186	
E	7887	2381	4613	4002	5606	3058	1691	13457	2918	1770	2096	1152	IL2
F	759	613	548	5607	638	906	5599	838	903	297	1920	820	
G	1469	1140	1032	9386	713	1382	790	918	932	414	940	867	
H	641	453	406	434	373	564	576	641	520	838	365	1752	

PID: 768 ASCII File: C:\Data\Janet\DATA.101 Acquired: 1/14/00 12:41:32 PM Plate Temperature: 18.8C (65.8F)

Repeat 1: Sample CPMA 3 10 30

media (2)

	1	2	3	4	5	6	7	8	9	10	11	12	
A	11528	23599	14112	21902	16491	37685	25691	26656	24069	7292	3300	2842	
B	10491	8452	7096	25211	17050	16284	22462	34170	25912	3332	4575	3085	IL4
C	17895	20503	23134	26573	25219	29279	28014	28629	33110	9776	8946	6757	
D	11673	14327	11235	16043	16337	20116	20756	22852	22574	4019	4655	3234	
E	7132	10749	6886	16340	15111	14913	18390	22295	21324	3026	2906	3575	
F	4206	4646	3732	3826	7226	11193	7645	9572	11013	3137	2944	3429	IL15
G	6095	8785	11154	11588	10324	11862	15590	13167	15580	6298	6453	4788	
H	3064	4559	4314	5141	5450	6323	5025	5569	5523	2945	3729	3667	

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PID: 769 ASCII File: C:\Data\Janet\DATA.101 Acquired: 1/14/00 12:52:45 PM Plate Temperature: 19.0C (66.1F)
Repeat 1: Sample CPMA

	1	2	3	4	5	6	7	8	9	10	11	12
A	10294	7586	8560	10449	9749	12401	9338	10945	9608	43029	43583	27433
B	8998	10684	9325	12365	8633	11091	12279	10795	9302	30904	36070	25700
C	11173	9642	15059	17337	13628	21905	12452	16447	14400	35074	32093	25854
D	6424	7246	10381	9541	8788	11971	10072	10202	8500	19361	18174	18169
E	27700	19322	22513	16995	13516	18409	23167	22938	24042	22692	25228	27529
F	15966	20779	12919	19796	17711	18681	26158	18851	21028	24673	23556	23585
G	21791	16222	18086	21551	17332	19624	16011	16499	21929	30220	25506	28443
H	13610	12515	10652	12960	9911	11914	12108	14552	11143	17344	16595	12382

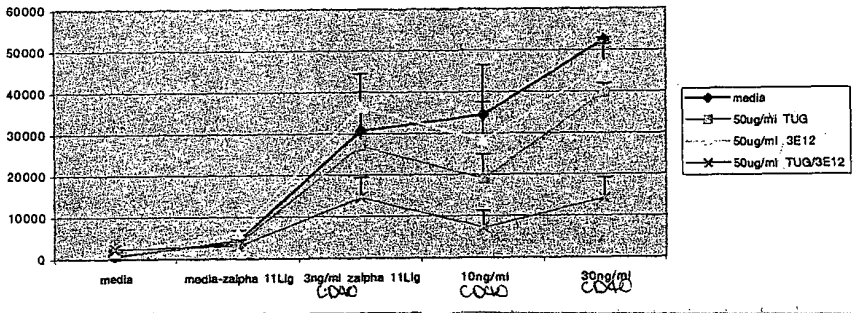
PID: 770 ASCII File: C:\Data\Janet\DATA.101 Acquired: 1/14/00 1:03:57 PM Plate Temperature: 19.1C (66.3F)
Repeat 1: Sample CPMA

	1	2	3	4	5	6	7	8	9	10	11	12
A	48273	52580	57146	70257	53615	75983	82489	61209	69718	30367	34902	34439
B	55969	62312	64224	70659	68051	78090	72177	76606	81429	40183	36706	35657
C	42012	52582	53539	68722	57248	66063	45775	68357	50093	25041	33151	36568
D	42202	49456	48245	57893	44011	58972	53410	60100	62761	23888	20213	20926
E	16163	12832	17072	19611	12122	17317	19862	19902	22365	23030	28302	25059
F	17807	24708	23824	28661	22854	25863	24487	30736	28209	26369	31701	28673
G	16454	15953	13412	15459	10185	16572	17145	15710	15691	31638	26039	27375
H	8221	14131	11714	15759	14181	24990	16216	19247	20620	15533	18518	13836

PID: 771 ASCII File: C:\Data\Janet\DATA.101 Acquired: 1/14/00 1:15:10 PM Plate Temperature: 18.7C (65.6F)
Repeat 1: Sample CPMA

	1	2	3	4	5	6	7	8	9	10	11	12
A	687	727	612	144	32	115	241	40	68	37	58	102
B	952	922	1039	96	75	182	205	116	65	153	57	96
C	1561	1737	1858	303	234	105	247	327	230	69	43	228
D	2151	1998	2400	852	123	395	103	71	272	35	75	82
E	1520	2130	1533	2585	1584	1572	1870	1614	1633	1090	1063	1191
F	7598	11786	10564	15733	13868	19751	18310	18749	22790	1697	1323	1344
G	3955	5901	4739	5514	7057	6141	6280	7388	7412	1146	1269	1619
H	3511	2295	1750	6761	5638	8984	16049	17163	12463	1460	1008	1268

7072.36 anti CD40 w/ titraring amounts of zalpha 11Lig w/wo Rx of TUG & 3E12 cells CD19 pos select from frozen PBMC



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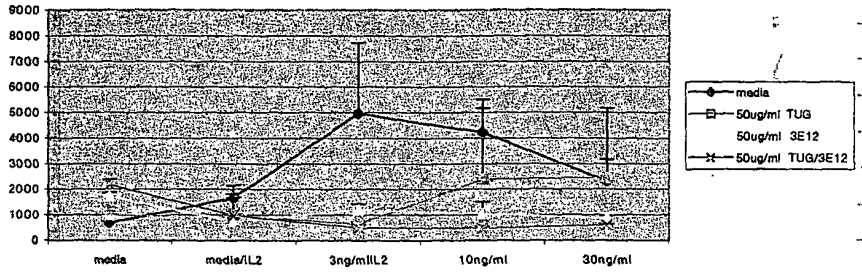
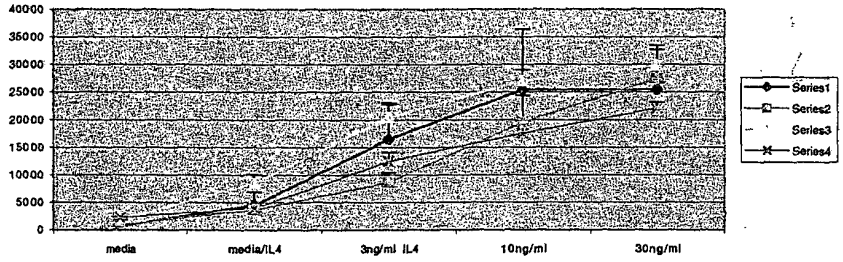
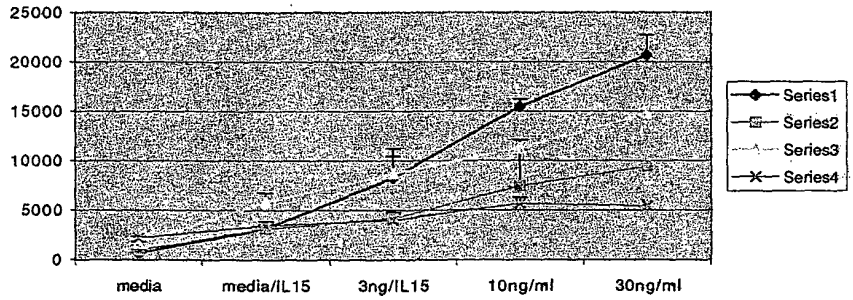
7072.36 anti CD40 w/ titraring amounts of IL12
w/wo Rx of TUG & 3E12
cells CD19 pos select from frozen PBMC



7072.36 anti CD40 w/ titraring amounts of IL14
w/wo Rx of TUG & 3E12
cells CD19 pos select from frozen PBMC



7072.36 anti CD40 w/ titraring amounts of IL15
w/wo Rx of TUG & 3E12
cells CD19 pos select from frozen PBMC



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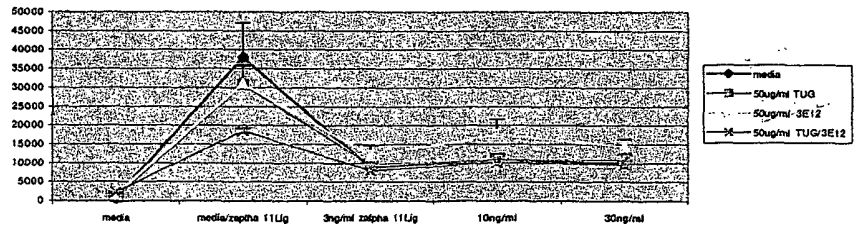
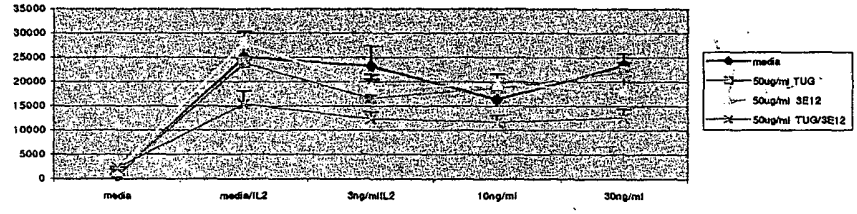
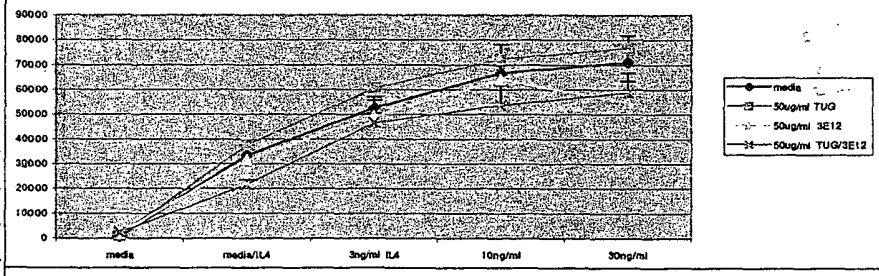
7072.36 anti IgM g w/ titrating zalpha 11Lig w/wo Rx TUG & 3E12
cells: CD19 pos select from frozen PBMC



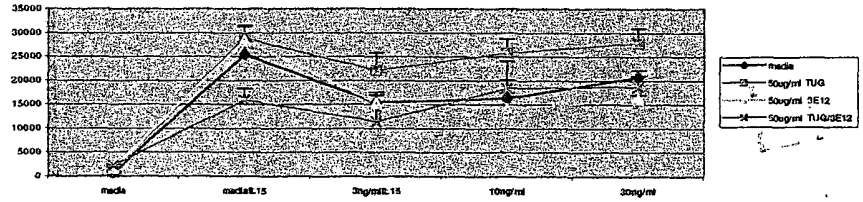
7072.36 anti IgM w/ IL2 w/ titrating zalpha 11Lig w/wo Rx TUG & 3E12
cells: CD19 pos select from frozen PBMC



7072.36 anti IgM w/ IL4 w/ titrating zalpha 11Lig w/wo Rx TUG & 3E12
cells: CD19 pos select from frozen PBMC



7072.36 anti IgM w/ IL15 w/ titrating zalpha 11Lig w/wo Rx TUG & 3E12
cells: CD19 pos select from frozen PBMC



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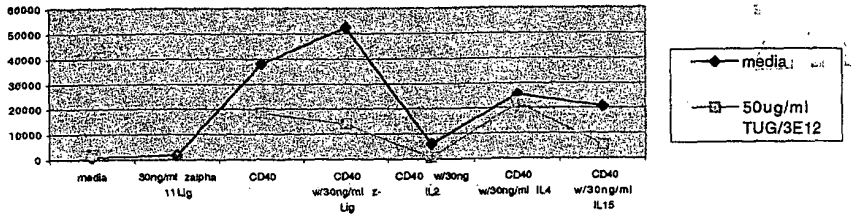
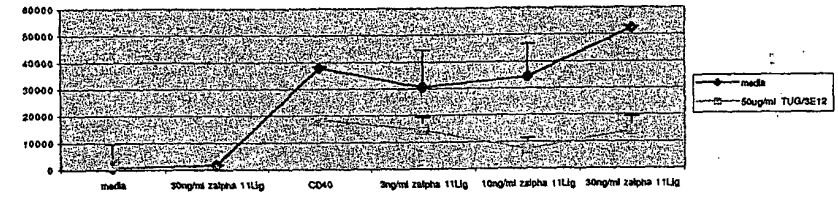
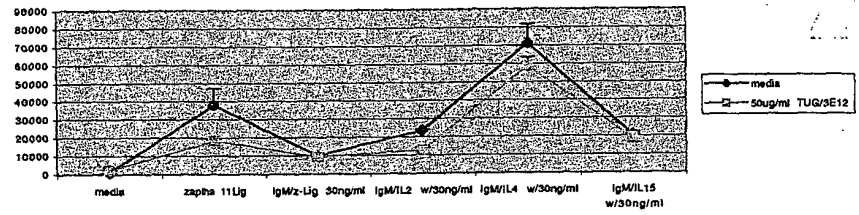
7072.36 CD40 w/ 30ng/ml of zalpha 11Lig, IL2, IL4, IL15 w/wo Rx of TUG & 3E12
cells CD19 pos select from frozen PBM



7072.36 CD40 w/ titraring amounts of zalpha 11Lig w/wo Rx of TUG & 3E12
cells CD19 pos select from frozen PBM



7072.36 anti IgM w/ IL2, IL4, IL15 w/ titrating zalpha 11Lig w/wo Rx TUG & 3E12
cells: CD19 pos select from frozen PBMC



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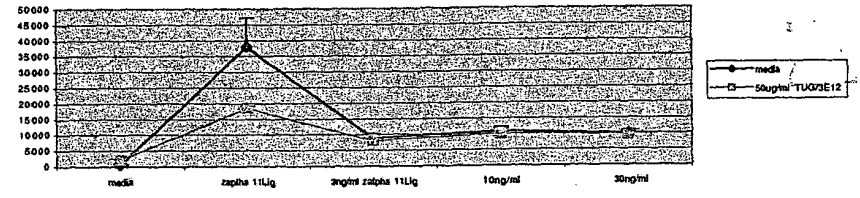
cells CD19 pos select from frozen PBMC



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Communication

Homodimerization of Interleukin-4 Receptor α Chain Can Induce Intracellular Signaling*

(Received for publication, April 26, 1996, and in revised form, August 5, 1996)

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The possible role of homodimerization events in intracellular signal transduction triggered by the bipartite human interleukin-4 receptor was addressed. We generated cell lines functionally expressing derivatives of the two receptor subunits α and γ, which allow for a specific and background-free experimental induction of intracellular homo- and heterodimers. A heterodimer of α and γ released an intracellular signal, whereas a γ-γ homodimer did not. Unexpectedly, we found the intracellular domain of interleukin-4 receptor α chain to evoke cell proliferation and activation of tyrosine kinase Jak1 as well as of transcription factor Stat6 upon homodimerization. Both recruitment of the common γ chain and activation of kinase Jak3 were shown to be dispensible for these processes.

Interleukin-4 (IL-4)[1] is a pleiotropic immune regulator with a pivotal role in certain allergic processes (1). The bipartite IL-4 receptor comprises the interleukin-4 receptor α chain (IL-4Rα) (2) and the common γ receptor chain (γc) (3, 4). Both receptor subunits belong to the cytokine receptor superfamily (5) and are shared by other cytokines; γc is also part of the receptors for IL-2, IL-7, IL-9, and IL-15 (6), and IL-4Rα contributes to the IL-13 receptor (7, 8).

Ligand-induced juxtaposition of the cytoplasmic domains of IL-4Rα and γc is believed to be a mandatory step in intracellular signaling which involves recruitment and activation of kinases Jak1 and Jak3 (9, 10), transcription factor Stat6 (11), and the adaptor molecule IRS-2 (12). However, the architecture of the IL-4R complex as well as the molecular mechanisms underlying the specificity of IL-4-induced signal transduction are to date poorly understood.

Making use of the strictly species-specific interaction of interleukin-4 with IL-4Rα chain, factor-dependent murine cells were rendered responsive to hIL-4 by expressing human IL-4Rα (2, 13–16). An implication of these results is the ability of human IL-4 to activate IL-4 receptor complexes containing either human or murine common γ chain, thus complicating an analysis of the composition of the signaling competent receptor subunit assembly.

In order to study the role of receptor chain dimerization events in signal release by the hIL-4R complex, we generated an expression system for receptor subunits that allowed us to experimentally induce specific and background-free intracellular hetero- and homodimerization.

Our results show that the juxtaposition of two intracellular domains of IL-4Rα can act as the trigger of specific signaling, including the activation of Jak1 and Stat6 and the induction of cell proliferation. Surprisingly, a hitherto assumed participation of the cytoplasmic portion of common γ chain and of γc-associated kinase Jak3 is not required.

MATERIALS AND METHODS

DNA Manipulations, Stable Transfection of Mouse Cells, and Detection and Quantification of Receptor Expression—Recombinant DNA work was performed according to standard procedures (17). The murine pre-B cell line Ba/F3 (18) has been described. BAF-4α-pγ, a Ba/F3 derivative expressing both subunits of the human IL-4R, is identical to BAF-4Rhγ (16).

Hybrid receptor genes were generated by polymerase chain reaction amplification of gene fragments from pKCR-pγ (16) encoding the epitope-tagged extracellular domain and transmembrane/intracellular domain of human γc and exchanging them for the corresponding fragments (*Bam*HI/*Xho*I or *Xho*I/*Hin*dIII) in pKCR-4α. The resulting expression plasmids pKCR-4α/γ and pKCR-pγ/α were cotransfected into Ba/F3 cells as described (16).

Surface expression of receptor constructs was assayed by reacting intact cells with antibodies X 14/38 (16, 19) or P5D4 (20) specific for the extracellular portions of recombinant hIL-4Rα or epitope-tagged human γc, respectively, and subsequent detection of bound antibodies by peroxidase-coupled secondary antibodies as detailed elsewhere (21). Briefly, 10^6 cells in a microtiter well were incubated on ice for 30 min with 5 μg of antibody in a volume of 50 μl of phosphate-buffered saline/3% bovine serum albumin. After washing twice, cells were resuspended in 100 μl of a 100 μg/ml solution of peroxidase-conjugated goat anti-mouse IgG (Dianova) and kept on ice for 30 min. Cell-bound secondary antibody was detected by transferring the cells to 50 μl of a solution containing 0.1 M Tris/HCl, pH 8.5, 2.5 mM 3-aminophthalhydrazide (Fluka), 400 μM *p*-coumaric acid (Sigma), 5.4 mM H_2O_2 and measuring elicited chemiluminescence using a MicroLumat LB 96P. Quantitation of surface-bound antibody molecules was achieved by relating the determined intensity of luminescence to a calibration series of samples containing known concentrations of peroxidase.

Cell Culture, Cytokines, and Proliferation Assay—Cell maintainance and preparation of hIL-4 and mutant Y124D has been described previously (16). Recombinant murine IL-4 was purchased from Sigma. Cytokine-induced proliferation of cell lines was measured by [^{3}H] thymidine incorporation into *de novo* synthesized DNA as described (16).

Immunoprecipitation, Immunoblotting, and Chemical Cross-linking—Samples of 3×10^7 cells were incubated at 37 °C for 10 min in 1 ml of RPMI containing no cytokine, 7 nM of IL-4, or 50 nM of antibody P5D4 and subsequently lysed as described (16). Cleared lysates were incubated with 1–5 μg of specific antibody. Antibodies used for immunoprecipitations were 4G10 (anti-phosphotyrosine, Upstate Biotechnology),

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[1] The abbreviations used are: IL, interleukin; hIL-4, human interleukin-4; hIL-4R, human interleukin-4 receptor; hIL-4Rα, human interleukin-4 receptor α chain; γc, common receptor γ chain; Jak, janus kinase; Stat, signal transducer and activator of transcription; α-, anti-.

[22) and anti-Jak1 rabbit serum (23). Immunocomplexes were ated from lysates with 50 μl of anti-mouse IgG-agarose or pro- Sepharose (Sigma) and assayed as described (16) using peroxi- ·jugated antibody RC20 (Transduction Laboratories) at a final ration of 0.1 μg/ml. Iodination of hIL-4, cross-linking of radioli- · cell-surface receptors, and analysis of immunoprecipitated com- by electrophoresis was carried out as described (19).

lysis of Stat Activation by Electrophoretic Mobility Shift Assay— cell extracts were prepared from cells stimulated with IL-4 or ly as described above by suspension of cell pellets in a buffer ing 20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EDTA, 20% l, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 leupeptin, 5 μg/ml, and 100 μM sodium *ortho*-vanadate followed e freeze-thaw cycles and centrifugation at 4 °C and 14,000 rpm nin. Supernatants equivalent to 10^5 cells were used for bandshift performed as described (24). As a probe, the Stat6-binding se- 5′-GTCAACTTCCCAAGAACAGAA-3′ derived from the human oter (25) end-labeled with polynucleotide kinase to a specific y of 8.000 cpm/fmol was applied. Supershifting of Stat6 contain- plexes was achieved by adding to the binding reactions before phoretic mobility shift assay 1 μg of a chicken antibody directed to acids 637–847 of murine Stat6.[2]

RESULTS AND DISCUSSION

intended to reconstitute in murine cells a functional kin-4 receptor complex activable exclusively by human which would not evoke any background signaling due to erence with the endogenous murine IL-4 receptor. To this we generated a pair of expression constructs encoding l receptor chains derived from hIL-4Rα and hγc with ally exchanged intracellular domains (Fig. 1*A*) and intro- it into the murine pre-B cell line Ba/F3.

clone expressing both 4α/γ and pγ/4α chimeras was d BAF-4α/γ-pγ/4α. The number of surface-expressed re- molecules per cell was determined in comparison with ine BAF-4α-pγ bearing both subunits of the authentic n IL-4R (Fig. 1*B*). As measured by the binding of specific odies recognizing the extracellular receptor domains, in ell lines surface expression of the receptor chain compris- intracellular domain of γc was considerably higher than f the subunit bearing the intracellular part of hIL-4Rα. ective of the "authentic" or "cross-over" composition of eterologous subunits, similar hIL-4 binding receptor com- could be formed in both cell lines as revealed by immu- cipitation of receptor chains cross-linked to radiolabeled (Fig. 1*C*).

test if the bipartite human IL-4R with exchanged cyto- ic domains was capable of transmitting specific signals to ell interior, we measured IL-4-induced cell proliferation. stimulated with hIL-4, BAF-4α/γ-pγ/4α cells expressing ombination of hybrid receptors, like BAF-4α-pγ cells, ed a proliferative response (Fig. 2*A*).

BAF-4α-pγ cells, hIL-4 mutant Y124D evoked 60% of the synthesis induced by wild type IL-4. We have previously n that this degree of reactivity is due to preferential in- tion of Y124D with murine γc (16). When assaying BAF- pγ/4α cells, we found, as earlier observed with human IL-4 ive cells (19), only 30% of wild type activity for hIL-4 nt Y124D. This result indicates that hIL-4 cross-over re- r, as anticipated and unlike its authentic counterpart, ides the formation of productive receptor complexes in- ng endogenous murine common γ chain.

mulation with hIL-4 resulted in equivalent patterns of ine-phosphorylated proteins in the two cell lines (Fig. 2*B*). intracellular domain of hIL-4Rα is a major substrate of d-induced phosphorylation as revealed by specific immu- cipitation (data not shown). Moreover, the modified hIL-4 tor was found to recapitulate hIL-4-specific activation of

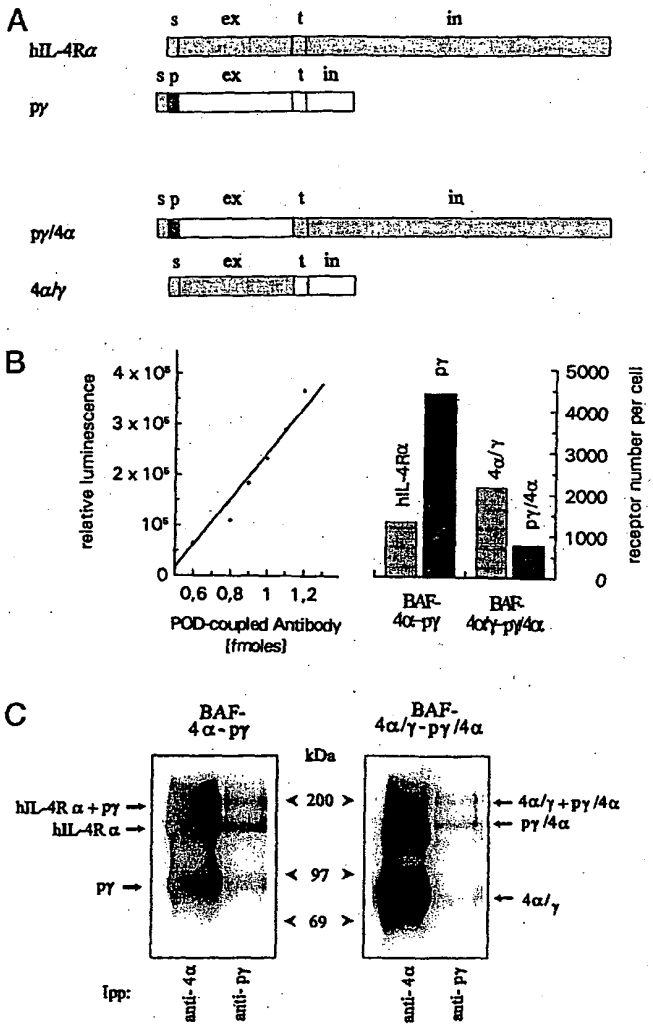

FIG. 1. **Generation and characterization of cell lines expressing hIL-4 receptor constructs.** *A*, schematic representation of bipartite authentic hIL-4 receptor expressed in BAF-4α-pγ cell (*top pair*) in hIL-4 cross-over receptor expressed in BAF-4α/γ-pγ/4α cells (*bottom pair*). *s*, signal peptide; *ex*, extracellular domain; *t*, transmembrane domain; *in*, intracellular domain; *p*, epitope tag recognized by antibody P5D4. *B*, analysis of receptor chain surface expression in BAF-4α-pγ and BAF-4α/γ-pγ/4α cells. Samples of 10^5 cells were reacted with antibodies directed to the extracellular domains of hIL-4Rα or epitope-tagged γc, respectively, washed, and stained with peroxidase-conjugated secondary antibody as described under "Materials and Methods." Numbers of bound enzyme molecules per cell equivalent to receptor chain copies were determined by quantification of elicited chemiluminescence and correlation of the signal intensity with a calibration series obtained by measuring luminescence produced by different known amounts of peroxidase under assay conditions. *C*, analysis of ligand-receptor complexes formed on BAF-4α-pγ and BAF-4α/γ-pγ/4α cells. After chemical cross-linking of ^{125}I-hIL-4 to the two cell lines, receptor complexes were immunoprecipitated using the indicated antibodies and subsequently resolved and visualized by SDS-polyacrylamide gel electrophoresis and autoradiography. Radioligand cross-linked receptor chains and complexes are marked with *arrows*.

janus kinases Jak1 and Jak3.

We next employed the model receptor system to address the individual roles of the IL-4 receptor subunits in signaling. The ligand and antibody binding properties of the functionally expressed receptor constructs enabled us to specifically induce all three possible intracellular receptor dimers (Fig. 3*A*). In BAF-4α/γ-pγ/4α cells, not only hIL-4-induced heterodimerization of the two intracellular receptor domains but surprisingly also antibody-mediated cytoplasmic homodimerization of hIL-4Rα via the extracellular P5D4 epitope tag lead to cell proliferation

. Heim and R. Moriggl, unpublished data.

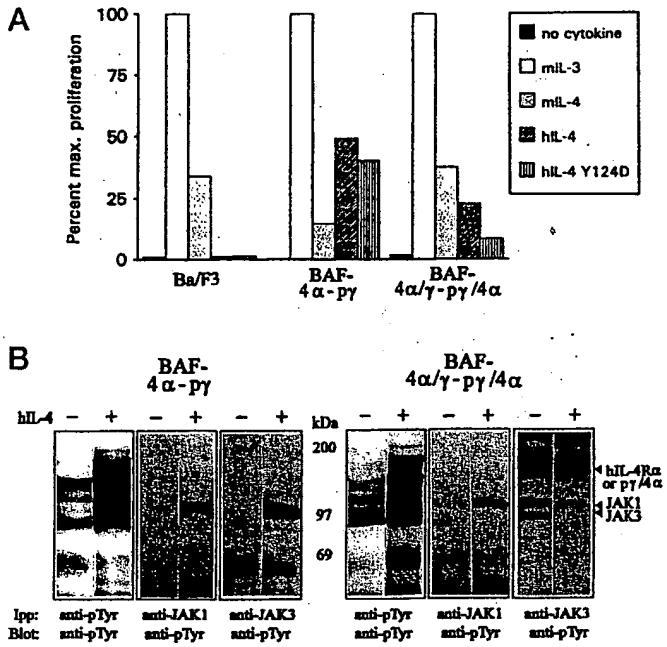

FIG. 2. **Cytokine-induced responses of BAF-4α-pγ and BAF-4α/γ-pγ/4α cells.** *A*, cytokine-induced proliferation of Ba/F3 cells and transfected derivatives. Cells were incubated with saturating concentrations of the indicated cytokines, and cell proliferation was determined by [^{3}H]thymidine uptake after 24 h. Radioactivity incorporated in response to murine IL-3 was set 100%. *B*, hIL-4-induced tyrosine phosphorylation in cell lines BAF-4α-pγ and BAF-4α/γ-pγ/4α. Lysates from hIL-4-stimulated or -unstimulated cells were subjected to immunoprecipitation with the indicated antibodies and Western blot analysis using anti-phosphotyrosine antibody for detection as described under "Materials and Methods." Positions of prominent phosphorylated proteins are marked by *arrows*.

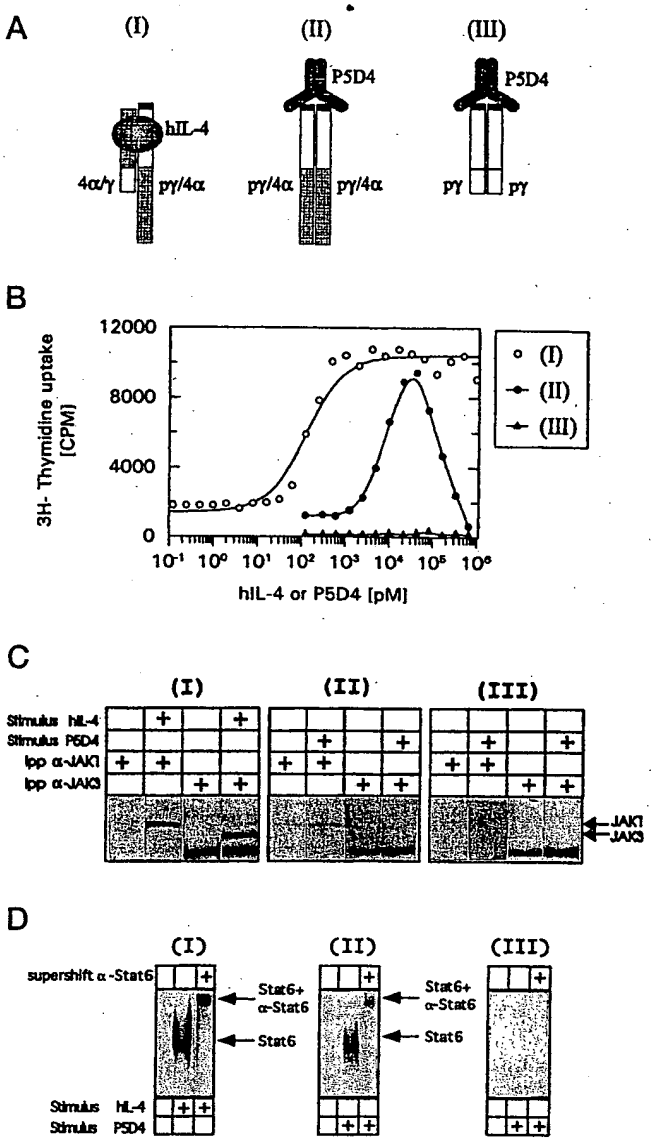

FIG. 3. **Directed cytoplasmic hetero- and homodimerization of hIL-4R subunits and resulting signal transduction.** *A*, schematic representation of stimulus-induced dimerization events. *I*, hIL-4-induced selective intracellular heterodimerization of hIL-4Rα and human γc in BAF-4α/γ-pγ/4α cells. *II*, intracellular homodimerization of hIL-4Rα via extracellular antibody and epitope tag in BAF-4α/γ-pγ/4α cells. *III*, antibody-induced homodimerization of γc in BAF-4α-pγ cells. *B*, cell proliferation evoked by the stimuli depicted under *A*. The respective cell lines were incubated with the indicated concentrations of hIL-4 (*I*) or antibody P5D4 (*II* and *III*) for 24 h before [^{3}H]thymidine uptake was measured. *C* and *D*, activation of Jak kinases (*C*) and activation of Stat6 (*D*) by the stimuli depicted under *A*. The respective cell lines were stimulated for 10 min with 10 nM hIL-4 (*I*) or 100 nM P5D4 (*II* and *III*). Cells were then lysed and subjected to immunprecipitations with anti-Jak antibodies and probing with anti-phosphotyrosine antibody (*C*) or to a band shift assay using a labeled probe derived from the Iε-promoter (*D*) as described under "Material and Methods."

(Fig. 3*B*). Antibody-induced homodimerization of γc intracellular domains in BAF-4α-pγ cells did not result in elevated DNA synthesis. The concentrations of hIL-4 and antibody P5D4, respectively, eliciting a proliferative response are in concordance with reported dissociation constants for the binding of hIL-4 to the high affinity hIL-4R of (100 pM) (26) and for the interaction between antibody P5D4 and its cognate epitope (100 nM) (21). The bell-shaped dose-response curve for the antibody-activity on BAF-4α/γ-pγ/4α cells indicates a blocking of receptor cross-linking by monovalent antibody binding at excess concentration and thus underscores our notion of P5D4-induced receptor homodimerization causing proliferation.

Comparing the activation of janus kinases known to be involved in IL-4 receptor complex function by hetero- or homodimerization, respectively (Fig. 3*C*), we found that antibody-induced intracellular homodimerization of hIL-4Rα results in tyrosine phosphorylation of Jak1 but not of Jak3. Homodimerization of intracellular γc does not lead to a detectable phosphorylation of Jak1 or Jak3, whereas the heterodimer of α and γ evokes the activation of both kinases. Activation of Stat6, as assayed by its property to bind to a cognate DNA sequence derived from the Iε-promoter, is induced not only by an intracellular heterodimer of α and γ but also by an α-α homodimer (Fig. 3*D*). From these results we conclude that both the cytoplasmic domain of γc and activated Jak3 are not mandatory for Stat activation and for the onset of a signaling cascade leading to cell proliferation. The essential trigger for the release of these events is rather the juxtaposition of two intracellular domains of IL-4 receptor α chain and the concomitant activation of Jak1 by tyrosine phosphorylation.

It is to date poorly understood how the common γ chain contributes to signaling mediated by different cytokine receptor complexes and how specificity of these receptors is achieved despite their sharing of a subunit. The only defined biochemical function of γc is the recruitment of Jak3 to the receptor complex (27). This very process, however, has been shown not to be essential for the specific activity of the IL-2 receptor; it can rather be replaced by artificially introducing Jak2 into the assembly (28). In this report we show that in the human IL-4 receptor system neither γc and Jak3 nor any substitute is required for the release of an intracellular signal if the intracellular domain of hIL-4Rα is experimentally homodimerized.

Our results raise new questions about the role of γc in the

on of the IL-4 receptor. The interaction of ligand with the ellular domains of both IL-4Rα and γc is necessary for aduced signal transduction, because mutant forms of defective in contacting γc fail to stimulate cell proliferation, 29). Functional properties of the intracellular domain in the activation of this particular receptor system have been addressed. Our data indicate that it is not involved release of intracellular signals specific for IL-4 and rt the notion of a more general role for γc in the formation signaling competent IL-4R and probably also other cytoeceptor complexes. In ligand-induced IL-4R activation, nction of γc and Jak3 could be the promotion of a transassembly of two or more copies of hIL-4Rα, a situation in turn would lead to specific intracellular signal transn. Alternatively, in the natural receptor complex, γcted recruitment of Jak3 might result in an activation of an event that in our model experiment is mimicked by xtaposition of two Jak1 molecules and serves as the r trigger for the various activities of hIL-4Rα. A more al version of such an interpretation of exchangeable Jaks hIL-4R complex would be the view that ligand-induced ellular apposition of several combinations of two Jak ules would suffice to evoke cell proliferation and the other ons observed. In this scenario, the major function of the ic receptor chain (here: hIL-4Rα) would be to provide ition sites for Stats and other downstream components pon Jak-driven activation mediate the particular effects 4. Directed homodimerization of γc does not result in ar activities because of its lack of recognition sites for stream signaling molecules. Also in line with such an nation would be the notion of cytokine receptor signal duction being relatively unselective and flexible in terms eractions between receptor chains and intracellular bindrtners. This would imply that the main event regulating icity in cytokine signaling is the recognition between re- and ligand and the thereby cross-linked combination of or subunits.

discriminate between the two principal explanations come with our results (involvement of receptor multimers in ral" hIL-4 receptor activation or low specificity of Jak ty combined with recruitment of signaling molecules by Rα via specific recognition sites), careful investigation of oichiometric subunit composition of the active hIL-4 re- complex and a mutational analysis of the cytoplasmic n of γc in this context are necessary. Also, the molecular details of Jak recognition, activation, and specificity in the hIL-4R assembly have to be addressed.

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THE INTERLEUKIN-2 RECEPTOR γ CHAIN: Its Role in the Multiple Cytokine Receptor Complexes and T Cell Development in XSCID

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KEY WORDS: interleukin 2, cytokine receptor, T cell development, XSCID, signal transduction

ABSTRACT

Interleukin 2 (IL-2), a T cell–derived cytokine, targets a variety of cells to induce their growth, differentiation, and functional activation. IL-2 inserts signals into the cells through IL-2 receptors expressed on cell surfaces to induce such actions. In humans, the functional IL-2 receptor consists of the subunit complexes of the α, β, and γ chains, or the β and γ chains. The third component, the γ chain, of IL-2 receptor plays a pivotal role in formation of the full-fledged IL-2 receptor; together with the β chain, the γ chain participates in increasing the IL-2 binding affinity and intracellular signal transduction. Moreover, the cytokine receptors for at least IL-2, IL-4, IL-7, IL-9, and IL-15 utilize the same γ chain as an essential subunit. Interestingly, mutations of the γ chain gene cause human X-linked severe combined immunodeficiency (XSCID) characterized by a complete or profound T cell defect. Among the cytokines sharing the γ chain, at least IL-7 is essentially involved in early T cell development in the mouse organ culture system. The molecular identification of the γ chain brought a grasp of the structures and functions of the cytokine receptor and an in-depth understanding of the cause of human XSCID. To investigate the mechanism of XSCID and development of

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gene therapy for XSCID, knockout mice for the γ chain gene were produced that showed similar but not exactly the same phenotypes as human XSCID.

INTRODUCTION

A variety of cytokines have been molecularly identified as soluble factors regulating the immune system, hematopoietic system, and other cell-cell interactions. They exert pleiotropic and redundant functions via their receptors expressed on multiple target cells (1). For understanding such cytokine actions, a series of cytokine receptors have also been molecularly identified and functionally characterized. Cytokine receptors are classified into at least five distinct families based on the structural characteristics of their extracellular and intracellular domains: the cytokine receptor superfamily, interferon receptor family, TNF receptor family, TGF-β receptor family, and IL-8 receptor family (2). The cytokine receptor superfamily is the largest family, containing at least 18 distinct receptor molecules, some of which may be shared among multiple cytokine receptors; the receptors for IL-3, IL-5, and GM-CSF contain the common β chain, and the receptors for IL-6, IL-11, OSM, CNTF, and LIF contain the common gp130. In essence, the common β chain and gp130 participate in increasing ligand-binding affinities and in ligand-induced intracellular signal transduction, suggesting that the sharing of receptor subunits may in part reflect the redundant functions of cytokines (2, 3).

The IL-2 receptors were originally classified into three isoforms, the high-, intermediate-, and low-affinity IL-2 receptors (4). Molecular characterization of the IL-2 receptor commenced with gene cloning of the α chain (IL-2Rα), which is unnecessary for intracellular signal transduction mediated by IL-2 (5–7). The second subunit of IL-2 receptor, β chain (IL-2Rβ), was then characterized as belonging to the cytokine receptor superfamily and as essential for the intracellular signal transduction (8). Introduction of IL-2Rα and IL-2Rβ genes induced the functional high-affinity IL-2 receptor in lymphoid cells but not in fibroblastoid cells, suggesting the possible existence of the lymphoid specific third component, the γ chain (IL-2Rγ), of the IL-2 receptor. Molecular identification of IL-2Rγ was achieved by coimmunopurification with IL-2Rβ, demonstrating that IL-2Rγ has the structural profile specific for the cytokine receptor superfamily, as does IL-2Rβ (9). Not only IL-2Rβ but also IL-2Rγ is essentially involved in the intracellular signal transduction, although their cytoplasmic domains do not contain any known effector function for signal transduction. However, associations of several effector molecules with their cytoplasmic domains have been revealed; in particular, novel tyrosine kinases Jak1 and Jak3 are physically bound with the serine-rich region of IL-2Rβ and the region containing the SH2 subdomains of IL-2Rγ, respectively (10).

Similar to the common β chain and gp130, IL-2Rγ was found to be shared with multiple cytokine receptors other than IL-2 receptor, such as receptors for IL-4, IL-7, IL-9, and IL-15 (10). Furthermore, the sharing of IL-2Rβ between IL-2 receptor and IL-15 receptor was recently found (11). All such receptor subunits shared by multiple cytokine receptors essentially participate in increasing ligand binding affinities and in ligand-induced intracellular signal transduction, suggesting that the sharing of receptor subunits may in part reflect the redundant functions of cytokines. The redundancy of cytokines may result in prevention of severe immunodeficiencies in mice targeted for cytokine genes; for example, gene-targeted mice for IL-2 and IL-4, both of which are capable of promoting T cell proliferation, carried normal numbers of T cells (12–14). However, knockout mice for IL-2Rβ were recently reported to show the deregulated T cell activation that resulted in autoimmunity (15). On the other hand, dysfunction of IL-2Rγ causes human XSCID, characterized by a profound defect of T cells (16). Consequently, the cytokines sharing IL-2Rγ should be implicated in the early T cell development.

THE IL-2/IL-2 RECEPTOR SYSTEM

Molecular Characterization of Receptor Subunits

At least three distinct subunits—IL-2Rα, IL-2Rβ, and IL-2Rγ—constitute IL-2 receptor complexes. The schematic structures of the human subunits are shown in Figure 1. The human IL-2Rα was originally detected using a monoclonal antibody (mAb) recognizing the Tac antigen expressed on activated T cells and leukemic cell lines carrying HTLV-I, and then it was molecularly cloned (5–7, 17). The human IL-2Rα gene is organized into eight exons spanning more than 35 kb and localized on chromosome 10p 14–15 (18, 19). The mature form of IL-2Rα, deduced from the nucleotide sequence, consists of 251 amino acid residues with no significant homology to known cytokine receptors except the recently identified α chain of IL-15 receptor (20) (Figure 1). The cytoplasmic domain of the IL-2Rα contains only 13 amino acid residues, which seems insufficient to harbor a signal transducing capacity. The second subunit β chain of IL-2 receptor (IL-2Rβ) was initially identified to be a 75-kDa cell surface glycoprotein, by affinity cross-linking experiments with radiolabeled IL-2 (21–25) and subsequently by mAbs specific for the human IL-2Rβ (26, 27). The complete cDNA clone encoding the human IL-2Rβ was isolated by expression cloning with the mAbs (8). The human genomic IL-2Rβ gene is partitioned into 10 exons, spanning 24 kb on chromosome 22q11.2–12 (28, 29). The

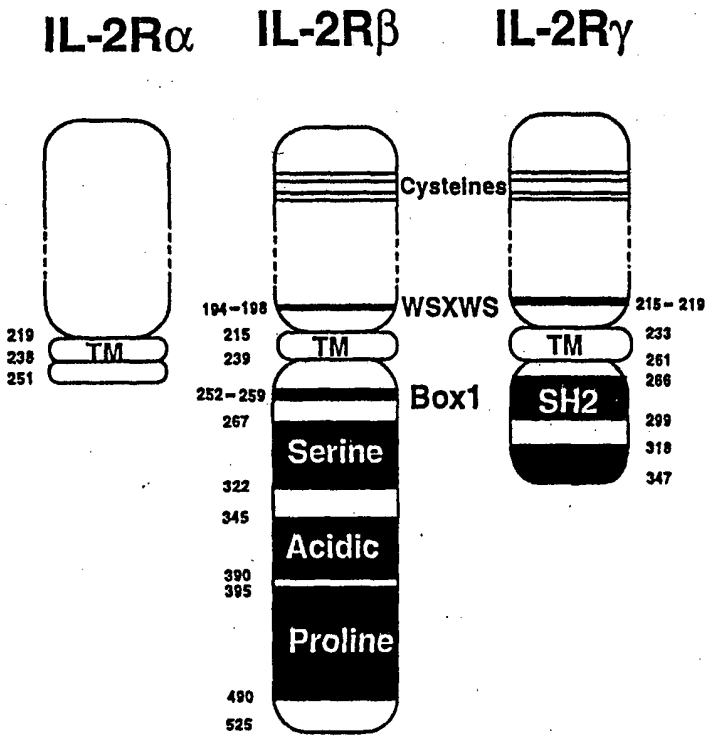

Figure 1 The schematic structure of the IL-2 receptor subunits, IL-2Rβ and IL-2Rγ. The numbers are amino acid positions from the amino terminals. The solid lines indicate the conserved cysteine residues. WSXWS indicates an amino acid sequence of tryptophan—serine—unconserved amino acid—tryptophan—serine. Box 1, Serine, Acidic, and Proline in the cytoplasmic domain of IL-2Rβ represent the box 1, serine-rich, acidic, and proline-rich regions, respectively. SH2 and C30 in the cytoplasmic domain of IL-2Rγ represent the SH2 subdomains and carboxyl terminal 30 amino acid residues, respectively.

mature form of IL-2Rβ consists of 525 amino acid residues. Characterized as a member of the cytokine receptor superfamily, IL-2Rβ has the common features of two pairs of the conserved cysteine residues near the amino-terminal and a sequence of tryptophan—serine—x(unconserved amino acid)—tryptophan—serine (WSXWS, WS motif) in the extracellular domain (30). The cytoplasmic domain of IL-2Rβ, consisting of 286 amino acid residues, contains unique regions such as the box 1, serine-rich, acidic, and proline-rich regions. The third subunit γ chain of IL-2 receptor (IL-2Rγ) was first detected by coimmunoprecipitation with IL-2Rβ prior to the IL-2Rβ gene cloning (31, 32).

TU11 mAb specific for the human IL-2Rβ precipitated a 64-kDa cell surface molecule distinct from IL-2Rα, together with IL-2Rβ in lysates of IL-2-treated cells expressing the high-affinity IL-2 receptor (31). The numbers of IL-2Rβ molecules on lymphoid transfectants with the IL-2Rβ gene usually exceeded sites of the intermediate-affinity receptor, which was known to contain IL-2Rβ but not IL-2Rα. The amount of the 64-kDa molecule coprecipitated with IL-2Rβ correlated well with the level of the intermediate-affinity IL-2 binding sites, suggesting the possibility that the 64-kDa molecule is IL-2Rγ (32). The 64-kDa molecule was purified by coimmunoprecipitation with IL-2Rβ, and its amino-terminal amino acid residues were determined. Based on the amino acid sequence, the complete cDNA clone encoding the 64-kDa molecule was isolated and demonstrated to be the cognate IL-2Rγ chain (9). The mature form of IL-2Rγ consists of 347 amino acid residues with sequences typical of the cytokine receptor superfamily such as IL-2Rβ. The cytoplasmic domain of IL-2Rγ, consisting of 86 amino acid residues, contains two subdomains of the Src homology region 2 (SH2). The full SH2 domain is known to contribute to the downstream signaling through its interaction with phosphotyrosine residues of various signal transducing effector molecules, but the two SH2 subdomains detected in IL-2Rγ are thought to be insufficient for such action (33). As described later, IL-2Rγ participates in formation of functional cytokine receptors not only for IL-2 but also for IL-4, IL-7, IL-9, and IL-15, and its dysfunction results in the occurrence of human XSCID.

Reconstitution of IL-2 Receptor Complexes

Expression of IL-2 receptor has been detected on hematopoietic cells and glioma cell lines but not on other nonhematopoietic cells including fibroblastoid cells and epithelial cells. Therefore, to investigate functional significances of the three distinct IL-2 receptor subunits, expression plasmids for human IL-2Rα, IL-2Rβ, or IL-2Rγ gene were stably transfected into the L929 fibroblastoid cell line, and the transfectant clones expressing various combinations of the receptor subunits were established. They were examined for their association, dissociation rate constants and affinities for IL-2 binding (9, 34) (Figure 2). Expression of IL-2Rα alone or of both IL-2Rα and IL-2Rγ showed low affinities (Kd = 10^{-8}) to IL-2 binding, and either IL-2Rβ or IL-2Rγ alone possessed undetectable affinities (Kd > 10^{-7} M) for IL-2 binding. The association rate constant with the αβγ heterotrimer complex was fourfold larger than that with the αβ heterodimer complex, and the dissociation rate constant was one fifth of that with the αβ heterodimer complex, resulting in two different types of high-affinity receptors with Kd of 10^{-11} and 10^{-10} M, respectively. Since the αβ heterodimer complex has no signal transducing ability for cell growth, it is referred to as the pseudo-high-affinity receptor. On the other hand, the βγ

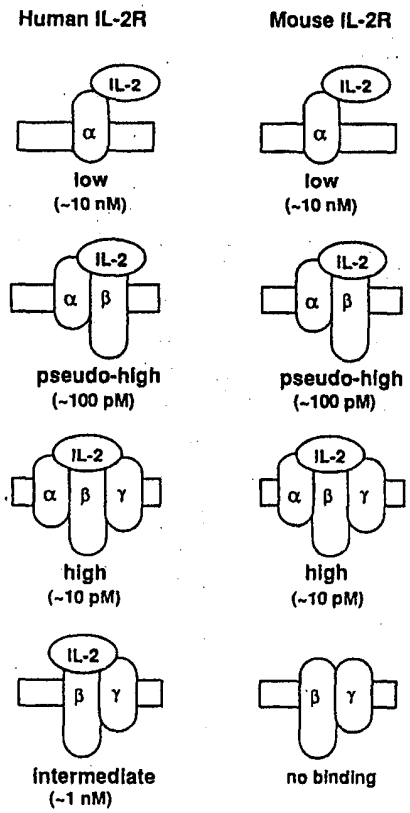

Figure 2 The human and mouse IL-2 receptor complexes and their affinities for IL-2 binding. The numbers in parentheses indicate IL-2 binding affinities.

heterodimer complex exhibited intermediate affinities (Kd = 10^{-9} M). The dissociation rate constants of the $\alpha\beta\gamma$ and $\beta\gamma$ complexes on lymphoid cells were generally much lower than those on fibroblastoid cells, suggesting that there is a significant difference between these two types of cells in regulating the dissociation of IL-2 from the receptors. The α chains of other cytokine receptors, such as IL-3, IL-5, or GM-CSF, and IL-6 or CNTF possess low affinities by themselves, but the heterodimers of the α chains with the β chains of such receptors form high-affinity receptors. These suggest a functional analogy between IL-2Rα and the α chains of other cytokine receptors.

IL-2 receptors reconstituted on fibroblastoid cells were also examined for their ability to transduce IL-2–mediated intracellular signals, as described later

in detail. The transfectants expressing the human $\alpha\beta\gamma$ and $\beta\gamma$ complexes of IL-2 receptor responded to IL-2 in terms of tyrosine phosphorylation of IL-2Rβ and induction of protooncogenes such as *c-myc, c-fos* and *c-jun* (35). On the other hand, the transfectants expressing the $\alpha\beta$ complex or the $\alpha\gamma$ complex showed unresponsiveness to IL-2. These indicate that both human IL-2Rβ and IL-2Rγ are essential and adequate for formation of functional IL-2 receptor complexes; IL-2Rα only increases the IL-2 binding affinity of the IL-2 receptor $\beta\gamma$ complex.

Similar reconstitution experiments of IL-2 receptor complexes were performed with expression plasmids for mouse receptor subunit genes, in an effort to characterize the difference between the human and mouse IL-2 receptor systems. The mouse $\alpha\beta\gamma$, $\alpha\beta$, and $\alpha\gamma$ complexes possess association and dissociation rate constants similar to those of human complexes, but the mouse $\beta\gamma$ complex has an undetectable affinity to IL-2 binding (36) (Figure 2). Such a difference in the IL-2 binding affinity of the $\beta\gamma$ complex between human and mouse was seen not only in transfectant cell lines but also in normal lymphocytes. Both human and mouse $CD8^+$ T cells and NK cells express the $\beta\gamma$ complex of IL-2 receptor, as described later. Although human $CD8^+$ T cells and NK cells exhibited IL-2 binding ability and IL-2 responsiveness, at least mouse $CD8^+$ T cells were unresponsive to IL-2 and incapable of binding IL-2 (36). Therefore, the three receptor subunits including IL-2Rα are indispensable for formation of the functional IL-2 receptor in the mouse system.

Expression of IL-2 Receptor Subunits

Expressions of IL-2Rα, IL-2Rβ, and IL-2Rγ on various populations of human peripheral blood cells were examined by staining with mAbs specific for each receptor subunit (37–39). IL-2Rγ expression was seen on all of the populations including $CD4^+$ T, $CD8^+$ T, $CD20^+$ B, $CD56^+$ NK cells, and $CD14^+$ monocytes. The granulocyte population was also positive for IL-2Rγ. On the other hand, IL-2Rα and IL-2Rβ were differentially expressed on these cell populations, although their expressions were enhanced by extracellular stimuli such as antigens and mitogens. $CD8^+$ T cells and $CD56^+$ NK cells significantly expressed IL-2Rβ, but little of IL-2Rα, while $CD4^+$ T cells expressed faint amounts of IL-2Rβ. Such a differential expression of IL-2Rβ on $CD4^+$ T and $CD8^+$ T cells reflects their respective IL-2 responsiveness, because the human $\beta\gamma$ complex forms functional receptor. In fact, $CD8^+$ T and NK cells freshly prepared from the peripheral blood showed a strong proliferative response to IL-2, whereas $CD4^+$ T cells required stimulation with macrophages for their IL-2 responsiveness (40, 41). It is of interest that CD16-$CD56^+$ NK cells in the human early pregnancy decidua express the high-affinity IL-2 receptor

consisting of the $\alpha\beta\gamma$ complex, suggesting that these NK cells may be activated in vivo (42).

Mouse splenic cell populations exhibited an expression pattern similar to that of the three receptor subunits to the human peripheral blood cell populations (43). Furthermore, mouse thymocytes were also examined for expression of the IL-2 receptor subunits and the α chains of IL-4 and IL-7 receptors that share IL-2Rγ as a common receptor subunit. The double negative ($CD4^-/CD8^-$) T cells, which are the most immature subset of T cells in thymus, were significantly positive for IL-2Rγ, IL-2Rα, and IL-7Rα. They were however almost negative for IL-2Rβ and IL-4Rα, predicting that double negative T cells express functional IL-7 receptor but little of functional IL-2 and IL-4 receptors (10). The double positive T cell subset contained a small population of IL-2Rγ positive cells and a large population of IL-4Rα positive cells but was negative for IL-2Rα, IL-2Rβ, and IL-7Rα, predicting that a small population of double positive T cells would express the functional IL-4 receptor, and that most of the double positive T cells would have no functional receptors for IL-2 and IL-7 (10).

IL-2Rγ is constitutively expressed on various populations of human and mouse hematopoietic cells, while expressions of IL-2Rα and IL-2Rβ chains are restricted to lymphocytes and monocytes/macrophages. Furthermore, IL-2Rα and IL-2Rβ expressions are different among cell populations, but they are known to be induced or enhanced within a day after stimulation with mitogens (37, 38). In contrast to IL-2Rα and IL-2Rβ expressions, IL-2Rγ expression on normal activated T cells was significantly suppressed by IL-2 stimulation. The IL-2-induced suppression of IL-2Rγ expression was also demonstrated by IL-2Rγ promoter-driven luciferase assays (44). Since IL-2Rγ is essential for the functional IL-2 receptor, the IL-2-induced suppression of IL-2Rγ expression may result in cessation of IL-2-dependent T cell growth. On the other hand, HTLV-I-infected human T cells are often established as IL-2-dependent cell lines, and such cell lines constitutively express the high-affinity IL-2 receptor (45). In these HTLV-I-infected T cell lines, IL-2 did not induce suppression of IL-2Rγ expression. In fact, a transacting transcriptional activator HTLV-I Tax was found to augment expression of IL-2Rγ; moreover, Tax nullified the IL-2–mediated suppression of IL-2Rγ expression (44).

IL-2Rγ IS A COMMON SUBUNIT FOR MULTIPLE CYTOKINE RECEPTORS

Sharing with the IL-4 Receptor

The cytokine receptor superfamily is known to include the common components for multiple cytokine receptors such as the β chain of receptors for IL-3, IL-5, and GM-CSF, gp130 of receptors for IL-6, IL-11, OSM, LIF, and CNTF, and the β chain of receptors for LIF, OSM, and CNTF (Figure 3) (2, 3). Expression of IL-2Rγ is detectable in a wide range of hematopoietic cell populations as distinct from IL-2Rα and IL-2Rβ expressions, allowing us to suppose that IL-2Rγ, apart from IL-2, serves as a multireceptor subunit. This supposition was suggested by the findings that human XSCID characterized by a T cell defect is caused by mutations of the IL-2Rγ gene (16), and that IL-2-deficient SCID patients and mice carry the normal phenotype of T cells (12–14, 46–48). IL-2Rγ was then predicted to be a common subunit of receptor complexes for IL-2 and other cytokines that may be necessary for early T cell development. To demonstrate such sharing of IL-2Rγ among multiple cytokine receptors, we and others applied two distinct procedures: one, blocking of cytokine functions by mAbs specific for the mouse IL-2Rγ, and two, reconstitution of cytokine receptors with transfection of IL-2Rγ and other cytokine receptor subunit genes. Candidate cytokines sharing the IL-2Rγ were expected to include cytokines affecting T cells. We established two types of mAbs, TUGm2 and TUGm3, specific for the mouse IL-2Rγ: TUGm2 can block the specific interaction between IL-2 and IL-2Rγ, and TUGm3 can precipitate IL-2Rγ cross-linked with IL-2. Using these mAbs, we have obtained evidence of IL-2Rγ sharing with receptors for IL-4, IL-7, and IL-9.

Initially identified as a B cell growth factor, IL-4 is known to possess the capacity to promote growth of T and mast cells (49). The α chain of IL-4 receptor (IL-4Ra) was identified as a 140-kDa molecule consisting of 800 amino acid residues, a member of the cytokine receptor superfamily (50). IL-4 responsive lymphoid cell lines expressed the high-affinity IL-4 receptor, whereas nonlymphoid COS-7 transfectant with the IL-4Rα gene expressed the IL-4 receptor with a lower affinity than that of the high-affinity IL-4 receptor on hematopoietic cells, suggesting that the high-affinity IL-4 receptor on lymphoid cells consists of a complex composed of at least IL-4Rα and another subunit. Consequently, IL-2Rγ was first examined for its sharing with the IL-4 receptor complex. TUGm2 significantly suppressed IL-4-dependent growth of CTLL-2 cells (51). Furthermore, in IL-4 binding assays, the binding of the high-affinity IL-4 receptor on CTLL-2 cells was significantly reduced by their treatment with TUGm2 (from 130 pM to 370 pM), although the IL-4 binding sites were unchanged (51). Since IL-2Rγ itself has no ability to bind IL-4 directly, IL-2Rγ was expected to form a complex with IL-4Rα. The direct participation of IL-2Rγ in the IL-4 receptor complex was then demonstrated by immunoprecipitation with another mAb specific for IL-2Rγ, TUGm3. CTLL-2 cells were treated with IL-4 and then chemically cross-linked, and their lysates were immunoprecipitated by TUGm3. IL-4-cross-linked IL-2Rγ was seen in the

immunoprecipitate, indicating the physical interaction between IL-4 and IL-2Rγ (51). All these results suggest the sharing of IL-2Rγ with IL-4 receptor (Figure 3). Similar results were obtained from the reconstitution experiments of IL-4 receptors by cotransfection of IL-2Rγ and IL-4Rα genes (52).

On the other hand, differential involvement of IL-2Rγ in formation of functional IL-4 receptors has been suggested; although TUGm2 significantly inhibited IL-4-induced proliferation of mouse BA/F3 cells, IC2 cells, and splenic B cells, it showed no effect on IL-4-induced expression of MHC class II molecules and CD23 on the cells (53). A possible physical interaction between IL-4 receptor and IL-13 has been suggested; IL-13 competitively inhibited binding of IL-4 to the functional IL-4 receptor (54, 55). Although IL-2Rγ is not shared with the functional IL-13 receptor, the sharing of IL-4Rα and an unknown component between receptors for IL-4 and IL-13 has been predicted (56, 57) (Figure 3).

Sharing with the IL-7 Receptor

Initially detected as a growth factor for pre-B cells derived from mouse bone marrow stromal cells, IL-7 was found to induce in vitro proliferation of T cells in combination with TPA stimulation, which proceeded in an IL-2-independent manner (58–60). Furthermore, IL-7-dependent proliferation of double negative thymocytes was seen in thymic organ cultures (61). Thus, the biological significance of IL-7 in B and T cell development was suggested. The α chain of human IL-7 receptor (IL-7Rα) was isolated by direct expression cloning strategy (62). The mature form of IL-7Rα consists of 439 amino acid residues with a calculated molecular weight of 49.5 kDa. The extracellular domain of IL-7Rα contains the features of the cytokine receptor superfamily, and the cytoplasmic domain with 195 amino acids in length does not contain consensus sequences for protein kinases. COS-7 transfectants with IL-7Rα expressed IL-7 receptors, of which the IL-7 binding affinity was significantly lower than that of the high-affinity IL-7 receptor expressed on lymphoid cells. These results suggest that the high-affinity IL-7 receptor on lymphoid cells consists of a complex composed of IL-7Rα and another receptor component. Thereby, IL-2Rγ was suspected to be a common receptor subunit shared with the IL-7 receptor. Although IL-2Rγ expressed on fibroblastoid transfectant cells were incapable of binding IL-7, IL-7-dependent proliferations of a mouse pre-B cell line, IxN/2b, and Con A-stimulated splenic cells were significantly suppressed by treatment with TUGm2, a blocking mAb specific for mouse IL-2Rγ (63). Simultaneous treatment with TUGm2 and A7R34, a mAb specific for mouse IL-7Rα, induced complete inhibition of the IL-7-dependent cell proliferation. The Scatchard analysis for IL-7 binding showed that IxN/2b cells express the high- and low-affinity IL-7 receptors, and their treatment with TUGm2 reduces the affinity of

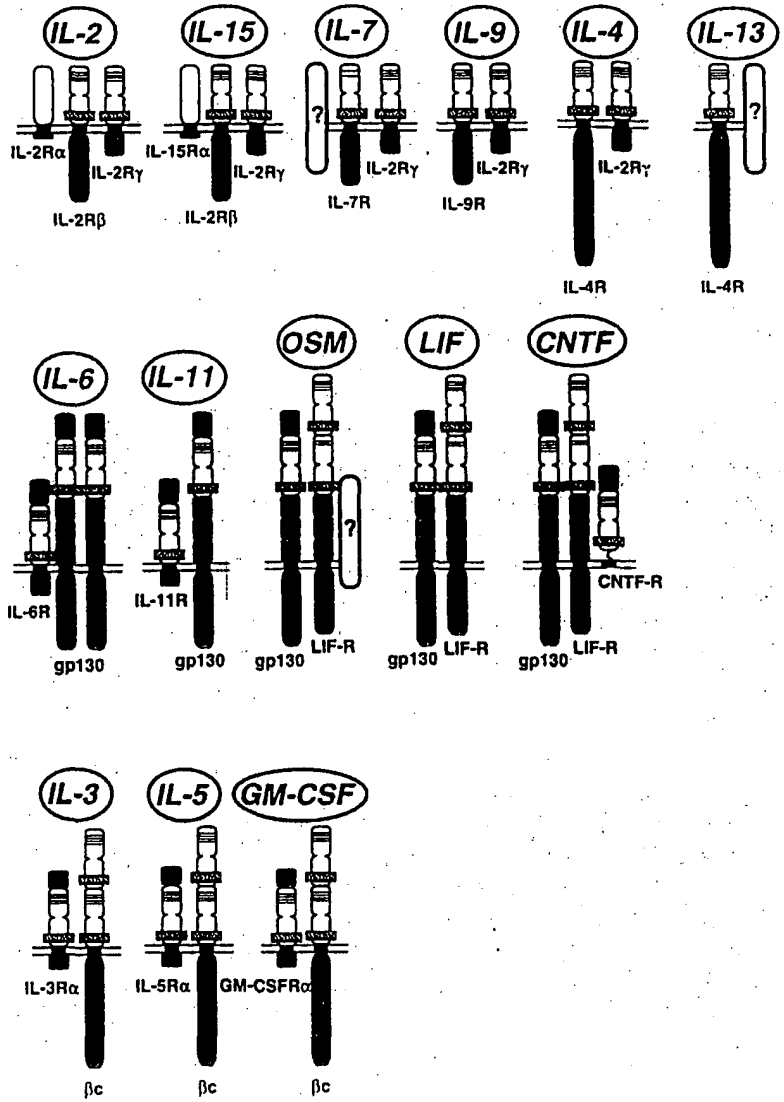

Figure 3 Sharing of the receptor subunits among multiple cytokine receptors.

the high-affinity IL-7 receptor from 79 pM to 255 pM without affecting the low-affinity receptor (63). Treatment of cells with both TUGm2 and A7R34 eliminated the high-affinity receptor, but the low-affinity receptor was unchanged. These results are similar to those of the IL-2/IL-2 receptor system, suggesting the possibility that the high-affinity IL-7 receptor consists of a tripartite complex composed of IL-7Rα, IL-2Rγ, and another unknown component that is expected to be involved in formation of the low-affinity receptor. An IL-7 receptor complex without IL-2Rγ may comprise the intermediate-affinity IL-7 receptor with a Kd of 255 pM. In a way similar to the IL-4 receptor, the physical association of IL-2Rγ with IL-7 was shown in the chemical cross-linking experiments. Radiolabeled IL-7-bound IxN/2b cells were chemically cross-linked and immunoprecipitated by TUGm3 (63). The immunoprecipitates apparently contained IL-2Rγ cross-linked with IL-7 in addition to other molecules, which may include IL-7-cross-linked IL-7Rα. All these observations indicate that IL-2Rγ is shared with the functional high-affinity IL-7 receptor (Figure 3). The reconstitution experiments of IL-7 receptors by cotransfection of IL-2Rγ and IL-7Rα genes also provided evidence of the IL-2Rγ sharing with IL-7 receptor (64).

Sharing with Other Cytokine Receptors

The sharing of IL-2Rγ with multiple cytokine receptors was further examined using IL-9 and IL-15, both of which have the ability to promote T cell proliferation. The α chain of IL-9 receptor (IL-9Rα) belongs to the cytokine receptor superfamily, and it consists of 483 amino acid residues (65). Its cytoplasmic domain contains a region highly homologous to IL-2Rβ, suggesting a functional similarity between IL-9Rα and IL-2Rβ. Since IL-2Rβ was known to form a complex with IL-2Rγ, IL-2Rγ was predicted to participate in formation of an IL-9 receptor complex. To assess this possibility, two mouse IL-9-responsive cell lines, MC/9 and CTLL-2, were examined for the effect of TUGm2 on their proliferation in response to IL-9. Their IL-9-dependent proliferations were almost completely inhibited by their treatment with TUGm2, suggesting the involvement of IL-2Rγ in formation of the functional IL-9 receptor (66). Unlike the other cytokine receptors sharing IL-2Rγ, however, Scatchard analysis showed no effect of TUGm2 on the affinity of IL-9 binding (66). On the other hand, the direct interaction of IL-9 with IL-2Rγ was shown by chemical cross-linking experiments. TUGm3 precipitated IL-9-cross-linked IL-2Rγ in addition to other molecules, which may include IL-9-cross-linked IL-9Rα (66). These observations indicate the sharing of IL-2Rγ with IL-9 receptor (Figure 3); they suggest that IL-2Rγ participates in the IL-9-mediated signal transduction but not in increasing the IL-9 binding affinity of the receptor. Another mAb specific for IL-2Rγ also reportedly induces inhibition of IL-9 responsiveness (67).

IL-15 was originally detected in supernatants of a simian kidney epithelial cell line, CV-1/EBNA, as an IL-2-like T cell growth factor mediating proliferation of IL-2-dependent cells. Recombinant IL-15 was found to induce proliferation of CTLL-2 and PHA-stimulated T cells, which also are responsive to IL-2 (11). The functional similarity between IL-15 and IL-2 was further accentuated by the finding that a mAb specific for IL-2Rβ significantly inhibits the biological activities of IL-15 (11, 68). Therefore, the interaction of IL-15 with the IL-2 receptor was then subjected to thorough investigation. The results revealed the sharing not only of IL-2Rβ but also of IL-2Rγ with the functional IL-15 receptor (69) (Figure 3). Recently, the α chain of IL-15 receptor was analyzed at the molecular level; it possesses a structure homologous to IL-2Rα (20) (Figure 3). The functional IL-15 receptor has been demonstrated to consist of a complex of IL-15Rα, IL-2Rβ, and IL-2Rγ, or a complex of IL-2Rβ and IL-2Rγ.

SIGNALING PATHWAYS FROM THE IL-2 RECEPTOR

Involvement of the Cytoplasmic Domain of IL-2Rβ

Most receptors for growth factors such as CSF-1, PDGF, EGF, and FGF possess intrinsic tyrosine kinase activities, whereas the ligand-induced activation of tyrosine kinases essentially contributes to intracellular signal transductions (70). Both IL-2Rβ and IL-2Rγ are indispensable subunits for the functional IL-2 receptor complex, which participate in increasing the IL-2 binding affinity and intracellular signal transduction (9, 35). The heterodimerization of the cytoplasmic domains of IL-2Rβ and IL-2Rγ generates intracellular signals for cell proliferation in T cells (71, 72), while the homodimerization of IL-2Rβ seems enough for signal transduction for cell proliferation in B cells (73). Both IL-2Rβ and IL-2Rγ belong to the cytokine receptor superfamily, of which the cytoplasmic domains, however, do not contain any consensus motifs of effector molecules for intracellular signal transduction, such as tyrosine kinases. However, they are reportedly associated with several nonreceptor-types of protein tyrosine kinases, whose activations mediated by ligand stimulation are thought to be important for signal transductions (74). The downstream signals mediated by the growth factors involve activations and cascade interactions of various signal transducing effector molecules, such as PI3 kinase, PLCγ, Grb2, SOS, Ras, GAP, Raf-1, MAP, kinase, and so on. These biochemical events are also known to be generated in the signaling pathways from various cytokine receptors including the IL-2Rβ and IL-2Rγ complex (10).

To elucidate the molecular basis for the signal transduction from the IL-2 receptor, various cytoplasmic deletion mutants of the IL-2Rβ and IL-2Rγ were

prepared and used for reconstitution of the receptor complex together with the wild type of each subunit. The mutants of human IL-2Rβ prepared had the serine-rich region, the acidic region, and most of the cytoplasmic domain deleted. The IL-2 receptor reconstitution studies with these IL-2Rβ mutants revealed that the serine-rich region of IL-2Rβ plays a crucial role in the IL-2–mediated signal transduction for cell growth and c-*myc* induction (75, 76). The acidic region of IL-2Rβ is associated with the Src type tyrosine kinases such as Lck and Fyn and probably Lyn (77, 78). The acidic region deletion mutant of IL-2Rβ failed to induce c-fos and c-jun expression in transfectant clones of a mouse IL-3-dependent pro-B cell line, BAF-B03, suggesting possible involvement of the Src-type tyrosine kinases in signaling for c-fos and c-jun induction (79). However, we have recently observed induction of c-fos and c-jun expression in transfectant clones of a mouse T cell line EL-4 with the acidic region deletion mutant of human IL-2Rβ, which (the transfectant clones) express the mutant IL-2Rβ and intrinsic IL-2Rγ (K Oda, H Asao, M Nakamura, and K Sugamura, unpublished observation). Therefore, there seems to be some element of controversy regarding the relationship between the inducing signal for c-fos and c-jun expression and the acidic region of IL-2Rβ. On the other hand, the serine-rich region of IL-2Rβ is associated with two types of tyrosine kinases such as Jak1 and Syk. The significance of Jak1 in signal transduction has not yet been defined, a point described later. Syk was activated in T cells rapidly after IL-2 stimulation, and its activation may have been involved in the c-myc induction (80).

Other effector molecules such as Shc and PI3 kinase were also associated with the cytoplasmic domain of IL-2Rβ (81, 82); in particular, PI3 kinase is reportedly associated with phosphorylated Tyr^{392} of IL-2Rβ (83). Activation of PI3 kinase was regulated by Fyn tyrosine kinase activated by IL-2 in IL-2-responsive T cells (84). Moreover, a recent study suggested that IL-2-induced activation of PI3 kinase leads to activation of the MAP kinase activator MEK in T cells, which is a novel signaling pathway (85). Shc is associated with Sos and acts upstream of Ras. In fact, Ras was previously shown to be activated in T cells stimulated by IL-2, and both the serine-rich and the acidic regions of IL-2Rβ were essentially involved in the activation of Ras (86).

The box 1 and box 2 regions were originally defined in the cytoplasmic domain of gp130 of IL-6 receptor and conserved among several cytokine receptors (87). The box 1 and box 2 of IL-2Rβ are located close to the transmembrane domain and in the serine-rich region, respectively. The replacement of Leu^{299} to Pro in the box 2 of IL-2Rβ rendered the IL-2 receptor incapable of cell growth signal transduction (88), and similarly, the substitution of Ala for ASP^{258} in the box 1 of IL-2Rβ markedly compromised such receptor function (89). These findings suggest the importance of the box 1 and box 2 regions of IL-2Rβ for receptor functions. The interaction of the box 1 with Jak1 tyrosine kinase is discussed later.

Involvement of the Cytoplasmic Domain of IL-2Rγ

The cytoplasmic deletion mutants of IL-2Rγ prepared had the carboxyl-terminal 30 amino acids, the SH2 subdomains, and the carboxyl-terminal 68 amino acids including the SH2 subdomains deleted. The reconstitution studies of IL-2 receptors with these mutants and IL-2Rβ demonstrated that the region containing the SH2 subdomains is essential for induction of cell growth and expression of three protooncogenes, *c-myc*, *c-fos*, and *c-jun*, mediated by IL-2 (35, 90). While the region containing the carboxyl-terminal 30 amino acids participates in induction of c-fos and c-jun, it does not for c-myc and cell growth (35, 90). These observations suggest the possible existence of at least two distinct signal transducing pathways from IL-2 receptor, one for induction of c-myc, which correlates with cell growth, and the other for induction of c-fos/c-jun. The region containing the SH2 subdomains is associated with Jak3 tyrosine kinase, the activation of which correlated with IL-2-induced cell growth (67, 90–93). In fact, transfectant clones of a mouse fibroblastoid cell line NIH3T3 with human IL-2Rβ and IL-2Rγ had little IL-2 responsiveness for cell growth, but when the clones were cotransfected with Jak3 gene, they became responsive to IL-2 for DNA synthesis, suggesting that at least Jak3 activation may contribute to signal transduction for IL-2–mediated cell growth (92).

Since the Jak3 association with IL-2Rγ is independent of the receptor complex formation with the other subunits IL-2Rα and IL-2Rβ, all the cytokines sharing IL-2Rγ are expected to induce activation of Jak3. Indeed, Jak3 is activated by stimulation with IL-4, IL-7, and IL-9 as well as IL-2 (67, 92, 93). Interestingly, not only Jak3 but also Jak1 is activated by stimulation with IL-4, IL-7, and IL-9, as well as IL-2 (67, 91, 92, 93). IL-15 is also expected to activate Jak1 and Jak3 because the IL-15 receptor complex contains both IL-2Rβ and IL-2Rγ. The association of Jak1 with IL-2Rβ was also demonstrated irrespective of their complex formation and IL-2 stimulation. Thereby, it can be predicted that Jak1 is associated with the α chains of receptors for IL-4, IL-7, and IL-9. Recently, we obtained evidence that Jak1 is directly associated with the α chains of receptors for IL-4, IL-7, and IL-9 as well as IL-2Rβ, and the Jak1 association with the receptor subunits was independent of ligand stimulation. Furthermore, using IL-2Rβ mutants in the box 1 region, which exists in the α chains of receptors for IL-4, IL-7, and IL-9 as well as other cytokine receptors, we demonstrated that the box 1 region of IL-2Rβ plays a critical role in Jak1 association. However, although transfectants with the box 1 mutant of IL-2Rβ lost the ability for Jak1 association and Jak1 activation mediated by IL-2, they

proliferated and showed activation of Jak3 in response to IL-2 (M Higuchi, H Asao, N Tanaka, M Nakamura, and K Sugamura, unpublished observations). These results indicate that Jak1 is dispensable for IL-2–mediated cell growth signaling, and activation of Jak3, which should be required for the growth signaling, is mediated independently of cross-phosphorylation with Jak1.

The Stat family proteins interacting with cytokine receptors at phosphotyrosine residues via their SH2 domains are phosphorylated by the Jak family tyrosine kinases (94). The tyrosine phosphorylated Stat proteins then form homo- or heterodimers through their SH2 domains and then migrate to the nucleus, where they act as activators for transcription of genes (95). IL-2 generally activated Stat5 in IL-2-responsive cells, and Stat3 only in PHA-activated peripheral blood cells (96–100). Stat5 and Stat3 are thought to be activated by Jak1 and/or Jak3 in IL-2-stimulated cells. In contrast with the IL-2/IL-2 receptor system, IL-4, which also activates Jak1 and Jak3, activates Stat6, which is distinct from Stat5 and Stat3 (101, 102). Such differential activation of the Stat proteins by the same Jaks may be regulated primarily by the specificity of the cytokine receptor directly interacting with the Stat proteins (95).

CAUSATIVE RELATIONSHIP BETWEEN IL-2Rγ AND HUMAN XSCID

Two-Step Diagnosis for Human XSCID

Human X-linked severe combined immunodeficiency disease (XSCID) occurs in as many as 50% of patients with primary SCID, which is characterized by severe impairment of humoral and cell-mediated immunity, which can be cured only by successful bone marrow transplantations (103, 104). Patients with XSCID have complete or profound deficiency of T cells but carry normal or slightly increased numbers of B cells. The putative gene for human XSCID is reportedly located on X chromosome q13, where the IL-2Rγ gene was mapped; furthermore, mutations of the IL-2Rγ gene derived from patients with XSCID were manifested (16). All the patients with XSCID thus far detected had mutations of the IL-2Rγ gene (10). The IL-2Rγ mutations included nonsense mutations, frameshift mutations by one and two base deletions, as well as deletions of exons, leading to truncations in the extracellular and cytoplasmic domains of IL-2Rγ. These results indicate the etiological relationship between mutations of IL-2Rγ and XSCID. Thereby, the genomic sequencing of the IL-2Rγ gene has been established as a definitive diagnostic procedure for human XSCID.

As described above, most peripheral blood cell populations of normal individuals express IL-2Rγ, indicating that expression of mutant IL-2Rγ chains

Table 1 Detection of IL-2Rγ mutations in patients with XSCID

Patient	Mutation type	Nucleotide change	Amino acid change	TUGh4 staining	Reference
1	splice/deletion	deletion of the exon 2	frameshift	−	(105)
2	deletion	7 nucleotides (284–291)	frameshift	−	(108)
3	deletion	15 nucleotides (580–594)	frameshift	−	(109)
4	deletion	GATT (830–833)	frameshift	−	our unpublished data
5	deletion	GA (971–972)	frameshift	+	(105)
6	deletion	large deletion	?	−	our unpublished data
7	nonsense	C (717) → T	Gln (235) → stop	−	(109)
8	nonsense	C (717) → T	Gln (235) → stop	−	(109)
9	nonsense	C (923) → A	Tyr (303) → stop	+	(109)
10	missense	C (481) → T	Ala (156) → Val	+	(105)
11	nonsense	C (690) → T	Arg (226) → Cys	−	(108)
12	nonsense	G (691) → A	Arg (226) → His	−	our unpublished data

can be screened with immunostaining of peripheral blood cells or EB virus-transformed B cells derived from patients with XSCID. The cells derived from 12 independent patients with XSCID were stained with anti-human IL-2Rγ mAb, TUGh4, resulting in negative staining for 9 patients (Table 1) (105–109). These results suggest that the IL-2Rγ immunostaining is useful as a simple and rapid diagnosis for most patients. Thus, we propose a two-step diagnosis for XSCID, the immunostaining with TUGh4 in the first step and the genomic sequencing of the IL-2Rγ gene.

Impairment of IL-2Rγ Function in XSCID

IL-2Rγ cDNA clones derived from three patients with XSCID were isolated, and the IL-2Rγ chains encoded by the mutant genes were examined for their ability to form the functional IL-2 receptor (105). The first patient lacked the second exon in IL-2Rγ mRNA, the second (AV mutant) showed Ala^{156} substitution to Val in the extracellular domain, and the third (tSH mutant) had a two-base deletion causing a frameshift of the coding region in the SH2 subdomains in the cytoplasmic domain. It can be easily anticipated that the first mutant will have no ability to form the functional receptor complex. Analysis was then carried out with the other two mutants, which were stably introduced into a unique human T cell line, $ED40515^-$ (105). This cell line is useful for analysis of the IL-2Rγ function because it expresses both IL-2Rα and IL-2Rβ, but little or no IL-2Rγ (110). The transfectants with either AV or tSH mutant showed no response to IL-2 in terms of cell proliferation and induction of

protooncogenes, unlike the transfectants with the wild IL-2Rγ gene. The AV mutant was further found incapable of forming the receptor complexes for IL-4 and IL-7 or for IL-2 (N Ishii, T Takeshita, M Higuchi, and K sugamura, unpublished data). The Ala^{156} of IL-2Rγ is thought to be located in the hinge region of two fibronectin type III-like domains, the N and C domains, as expected for the cytokine receptor superfamily. The transfectants with tSH mutant showed significant binding of IL-2, which is consistent with the previous study that the cytoplasmic domain of IL-2Rγ does not affect the IL-2 binding affinity of the receptor (105). Since the tSH mutant lacks a part of the SH2 subdomains, which is essentially involved in the intracellular signal transduction, it should lose the signal transducing function. Other patients with XSCID carrying mutations in the cytoplasmic domain of IL-2Rγ were also shown to be incapable of forming the full-fledged functional IL-2 receptor complex (67).

Normal or increased numbers of B cells and hypogammaglobulinemia are generally detected in patients with XSCID. Since the B cells in XSCID are mostly surface IgM-positive, they are supposed to be incapable of secreting immunoglobulin. Such impairment of Ig class-switching and final maturation of B cells in XSCID may also result from dysfunction of IL-2Rγ on B cells. This stems from the facts that carrier females for mutant IL-2Rγ genes were demonstrated to carry mature B cells with nonrandom inactivation of the X chromosome, and various B cell clones derived from three patients with XSCID were found to utilize the biased repertoire of the J_H segments (107, 111, 112).

The complete dysfunction of IL-2Rγ such as failure of the ligand binding or inability to perform signal transduction results in the typical phenotypes of XSCID as characterized by T and NK cell defect. However, atypical cases of XSCID have also been reported; one case showed a normal number of $CD4^+$ and $CD8^+$ T cells in the periphery, but their function was impaired, and the other case showed a reduced number of T cells and normal number of NK cells (67, 113). In the former patient, the IL-2Rγ gene was transcribed into two different mRNAs, one of which encodes the nonfunctional IL-2Rγ rather abundantly as compared with the other, which encodes the functional IL-2Rγ despite a mutation of Asp^{39} to Asn. The small amount of the functional IL-2Rγ was thought to be adequate for T cell development but not for activation. The latter patient had a mutation of Leu^{291} to Glu in the cytoplasmic domain of IL-2Rγ, although the mechanism leading to the atypical phenotypes of XSCID has not yet been clarified.

Involvement of IL-7 in Early T Cell Development

The mutations of the IL-2Rγ gene result in XSCID, of which the typical feature is a profound T cell defect in the thymus and periphery. Since IL-2Rγ is shared among receptors for at least IL-2, IL-4, IL-7, IL-9, and IL-15, these cytokines

may include a responsible cytokine(s) for early T cell development. In this consideration, the IL-2-deficient patients and the knockout mice for IL-2 and IL-4 genes provided us with important information—that neither IL-2 nor IL-4 are essential for T cell development because these patients and knockout mice have mature T cells in normal ranges in the periphery (12–14, 46–48). On the other hand, IL-7 reportedly affects growth of double negative T cells (61). We then asked, using mouse thymic organ cultures, whether IL-7 is necessary for early T cell development. When the most immature thymocytes—$Pgp1^+$/$c\text{-}kit^+$ pro-T cells with $CD4^-8^-$ phenotype, sorted from Day 15 fetal thymus—were cultured in lobes of fetal thymus pretreated with deoxyguanosine to eliminate preexisting lymphocytes, $CD4^+$ and $CD8^+$ T cells were detected 7–10 days later. However, simultaneous addition of blocking mAbs specific for IL-7Rα (A7R34) and IL-2Rγ (TUGm2) into the culture completely inhibited development of the double-negative $Pgp1^+$/$c\text{-}kit^+$ pro-T cells into double-positive T cells, indicating that IL-7 plays a critical role for early T cell development at least in the mouse thymic organ culture system (114). These results suggest that the IL-2Rγ mutants in XSCID accompany the dysfunction of IL-7, resulting in the defect of T cell development. As described above, the AV mutant of IL-2Rγ derived from the patient with XSCID showed no ability for binding of IL-7 as well as IL-2 and IL-4. However, there is a dissimilarity between the phenotypes of mice with IL-7 dysfunction and human XSCID. Human XSCID has a normal or increased number of B cells, while in vivo blocking of mouse IL-7 function by antibody treatment induces significant reduction of B cells in addition to T cells (114, 115). Similarly, knockout mice for IL-7 or IL-7Rα gene showed the profound defect of T and B cells (116, 117). This inconsistency has to be resolved in the future to define whether or not XSCID is solely attributable to the dysfunction of IL-7.

ANIMAL MODEL FOR HUMAN XSCID

An animal model of human XSCID is useful for investigating the occurrence of XSCID caused by mutations of the IL-2Rγ gene and development of gene therapy for human XSCID. Knockout mice for the IL-2Rγ gene were recently developed, and they showed significant reduction of T, B, and NK cells (118, 119). Their phenotypes, which are not exactly identical to those of human XSCID with regard to B cell development, are quite similar to those of IL-7-dysfunctional mice. They are completely null for expression of IL-2Rγ on cell surface. However, since a couple of cases of human XSCID lack the cytoplasmic region of IL-2Rγ, they are expressed on cell surface, and they work for ligand binding but not signal transduction. Therefore, we developed, by means of gene targeting, mice expressing IL-2Rγ but lacking its cytoplasmic

domain. Our mutant mice also showed profound loss of T and B cells, and no NK cells, but in contrast to the null mutant mice, the number of monocytes was increased in our mutant mice (120). Interestingly, they had $CD34^+$/c-kit^+/$Sca1^+$ hematopoietic stem cells in spleen more than ten times as often as the control, and they carried lymphoadenopathy of celiac lymphnodes. Such increase of the stem cells was seen in our mutant mice but not in the null mutant mice, suggesting that traps of the cytokines sharing IL-2Rγ might be involved in this phenomenon (121). In humans, patients with the primary and secondary immunodeficiencies have a high risk of developing lymphomas; therefore, the lymphoadenopathy observed in our mutant mice may represent a prelymphomatous state (121). Our mutant mice as well as the null mutant mice share similar phenotypes of profound loss of T, B, and NK cells, suggesting that these mutant mice are deficient for humoral, T cell–mediated, and NK cell–mediated immunities. Since the SCID strains of mice established previously have NK cell activities, the IL-2Rγ mutant mice may serve as a more desirable SCID mouse model.

FUTURE DIRECTIONS

The discovery and molecular characterization of the third component of IL-2 receptor, IL-2Rγ, have brought us a tremendous amount of knowledge that helps us to understand the structure and signal transducing functions of various cytokine receptors. These developments have made especially great contributions toward elucidation of the molecular mechanisms of human XSCID occurrence. The cytokines sharing IL-2Rγ induce their pleiotropic and redundant functions. As expected, IL-2Rγ interacts with Jak3, which can be activated by stimulation with all the cytokines sharing IL-2Rγ. Moreover, it is noteworthy that the α chains of cytokine receptors sharing IL-2Rγ, albeit different from each other, interact with the same effector molecule Jak1. Thereby, the redundancy of cytokine actions possibly resulted, in part, from sharing of receptor subunits, and in part, from sharing of the same effector molecule irrespective of receptor subunit sharing. Little is known about the signal transduction for the pleiotropy of IL-2 functions; however, the cytoplasmic domain of IL-2Rβ interacts with several other effector molecules such as Lck, Fyn, Syk, PI3 kinase, and Shc/Grb2/Sos/Ras, and Stats. Furthermore, the region containing the carboxyl-terminal 30 amino acids of the IL-2Rγ cytoplasmic domain, which is essentially involved in signal transduction for induction of c-fos and c-jun, is expected to interact with a certain effector molecule(s). Some of these effector molecules associated with the IL-2 receptor complex may contribute to the pleiotropic function of IL-2. To demonstrate the signaling pathways for the pleiotropic and redundant functions of the cytokines sharing IL-2Rγ, we need to further investigate the downstream events of these effector molecules associated with the receptors.

The causative relationship between human XSCID and mutations of the IL-2Rγ gene has been demonstrated. Knockout mice for IL-2Rγ showed similar phenotypes to those of human XSCID, although their phenotypes were not completely the same. The difference is that B cells are significantly reduced in mouse XSCID but not in human XSCID. Similarly, the mechanism of the T cell defect in XSCID can be also explained by dysfunction of IL-7 sharing IL-2Rγ in the mouse thymic organ culture system, but dysfunction of IL-7 in mouse is known to cause the reduction of B cells. Hence, although it is strongly suggested that the IL-7 dysfunction directly leads to occurrence of XSCID in humans, it has become important to resolve the difference in usage of IL-2Rγ for B cell development between human and mouse. Apart from the precise mechanism of XSCID occurrence, the knockout mice for IL-2Rγ will provide a useful tool for the development of gene therapy for human XSCID.

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